Population structure and

host-parasite interactions in the *Daphnia longispina* hybrid complex

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Population structure and host-parasite interactions in the *Daphnia longispina* hybrid complex

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Declaration of contributions as a co-author

In this dissertation, I present the work of my doctoral research from January 2009 to August 2011. I participated in field sampling, did large part of molecular work in the laboratory, and conducted all the data analysis. I also led the paper writing for first three papers/manuscripts (Chapter 2, 3 and 4) and took part in paper writing in the Chapter 5. Dr. Sabine Gießler and Dr. Adam Petrusek assisted and revised the some of the chapters, and Dr. Justyna Wolinska assisted and revised all the chapters and led the paper writing in the Chapter 5. The work has resulted in two publications (Chapter 2 and 5) and two submitted manuscripts (Chapter 3 and 4). The thesis consists of four manuscripts and is supplemented by appendices:

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SUMMARY

Natural hybridization, the interbreeding of species, can contribute to understanding the processes of maintaining biodiversity. There are different theories to explain the coexistence of hybrids with their parental species. Some models assume that hybrids are temporarily more or equally successful as parental taxa in a specific environment; but even in case of lower fitness of hybrids, a dynamic equilibrium between natural selection against hybrids and dispersal explains the maintenance of hybrid zones. Although the long-term significance of hybridization in animals is poorly understood, it is common among cyclical parthenogens, especially in zooplankton species of the genus *Daphnia*.

In the first part of my thesis (Chapters 2-4), I investigated the population structure of *Daphnia longispina* assemblages under different selection pressures. First of all, in Chapter 2, I detected a nearly perfect correspondence in the assignment of *Daphnia* individuals to different parental and hybrid taxa based on microsatellite markers (15 loci) when examining reference clones which had been previously classified by different markers (allozymes, mtDNA). This allowed me to identify species and different hybrid classes from field samples by microsatellite markers alone and their assignment was verified by a set of statistical approaches (Factorial Correspondence Analysis and two Bayesian methods). Secondly, by applying microsatellite markers on *Daphnia* samples isolated from eight different lakes, I explored the dynamics of the hybridizing system (Chapter 2). Within taxa, replicated genotypes were of clonal origin but clonal lineages rarely persisted in subsequent years suggesting that populations must go through sexual reproduction to be re-established in spring, from sexually produced diapause eggs. In addition, I also observed a complete replacement of taxa between two spring seasons (Chapter 2). Such a year-to-year taxon replacement has not been reported for the *D. longispina* complex before. I additionally detected that the genotypic diversity is lower in hybrids than in parental species (Chapters 2 and 3), supporting the

existence of reproductive incompatibilities between the parental genomes. Thirdly, in order to understand the impact of cyclically parthenogenetic reproduction on populations, I explored the changes in taxon and clonal composition of *Daphnia* populations, across time (generation-to-generation) and space (between sampling stations), during a period of seasonal environmental change (Chapter 3). I observed that clonal diversity increased with time, as a few dominant clones were replaced by a higher number of less common clones. I assumed that a loss in selective advantage for the dominant clones may have been due to parasite selection acting in a negative frequency-dependent manner. Therefore, in Chapter 4, I investigated the possibility of parasite-mediated selection in *D. longispina* populations. I found significant differences in clonal composition between random and infected parts of the host population. This suggests that parasite-driven selection might operate in natural *Daphnia* populations, as parasites influence the clonal structure of host population.

In the second part of my thesis (Chapters 5), I investigated how host-parasite interactions could be altered by predation. Specifically, I tested the potential costs of simultaneous prey exposure to enemies from different functional levels (i.e. predators and parasites). I found that the proportion of successful infections and the number of parasite spores were higher among defended (against predators) than undefended *Daphnia*, demonstrating a previously unknown and environmentally relevant cost to inducible defences. These results enhance our understanding of how epidemiology can be integrated into the concept of phenotypic plasticity.

ZUSAMMENFASSUNG

Natürliche Hybridisierung, also die Kreuzung zwischen verschiedenen Arten, kann dazu beitragen Prozesse zu verstehen, die Biodiversität aufrechterhalten. Es gibt unterschiedliche Theorien, um die Koexistenz von Hybriden mit ihren Elternarten zu erklären. Manche Modelle gehen davon aus, dass Hybride zeitweise erfolgreicher oder zumindest genauso erfolgreich sind wie ihre Elternarten. Aber auch wenn Hybride eine geringere Fitness haben, erklärt ein dynamisches Gleichgewicht zwischen natürlicher Selektion gegen Hybride und deren erneuter Ausbreitung, dass Hybridzonen erhalten bleiben. Obwohl die langfristige Bedeutung von Hybridisierung bei Tieren unzureichend verstanden wird, ist Hybridisierung unter zyklisch parthenogenetisch reproduzierenden Arten weit verbreitet, im Zooplankton besonders bei Arten der Gattung *Daphnia*.

Im ersten Teil meiner Doktorarbeit (Kapitel 2-4) untersuchte ich die Populationsstruktur von taxonomisch gemischt zusammengesetzten *Daphnia longispina* Gemeinschaften unter verschiedenem Selektionsdruck. Zuallererst fand ich bei der Zuordnung von Daphnienindividuen zu unterschiedlichen Arten und Hybridtypen eine nahezu perfekte Übereinstimmung, wenn Referenzklone, die zuvor bereits mit anderen Markersystemen klassifiziert worden waren (Allozyme, mtDNA), mit Mikrosatellitenmarkern (15 Loci) identifiziert wurden. Dies erlaubte mir, Arten und verschiedene Hybridklassen in Freilandstichproben allein anhand von Mikrosatellitenmarkern zu identifizieren. Die Zuordnung zu den unterschiedlichen taxonomischen Gruppen wurde durch ein Set verschiedener statistischer Verfahren verifiziert (Factorial Correspondence Analysis und zwei Bayes'sche Verfahren). Zweitens untersuchte ich die Dynamik des Hybridsystems, indem ich Daphnienproben, die aus acht verschiedenen Seen isoliert worden waren, mit Mikrosatellitenmarkern typisierte (Kapitel 2). Innerhalb der Taxa waren replizierte Genotypen klonalen Ursprungs. Klonlinien überdauernden aber selten aufeinanderfolgende Jahre. Das

impliziert, dass sich die Populationen sexuell fortpflanzen und dass Populationen durch Schlüpfen aus sexuell produzierten Dauereiern im Frühjahr wieder hergestellt werden. Ferner beobachtete ich im Frühling der beiden untersuchten Jahre einen kompletten Wechsel zwischen zwei Taxa (Kapitel 2). Solch ein kompletter, jährlich alternierender Wechsel wurde im *D. longispina* Komplex bisher noch nie beschrieben. Außerdem fand ich, dass die genotypische Diversität bei Hybriden niedriger als bei den Elternarten ist (Kapitel 2 und 3), was die Existenz reproduktiver Inkompatibilitäten zwischen den Elterngenomen unterstützt. Drittens untersuchte ich die Veränderung in der Taxon- und Klonzusammensetzung von Daphnienpopulationen über Zeit (von Generation zu Generation) und Raum (zwischen Probestellen) während einer Phase saisonaler Umweltänderung, um zu verstehen, wie sich zyklisch parthenogenetische Vermehrung in Populationen auswirkt (Kapitel 3). Ich beobachtete, dass die klonale Diversität mit der Zeit anstieg, wobei wenige, dominante Klone durch eine größere Anzahl seltenerer Klone ersetzt wurden. Ich nahm an, dass ein abnehmender Selektionsvorteil der dominanten Klone daher resultieren könnte, dass Selektion durch Parasiten in einer negativ häufigkeitsabhängigen Weise wirkt. Deshalb untersuchte ich in Kapitel 4, ob Selektion in *D. longispina* Populationen durch Parasiten ausgelöst werden könnte. Ich fand signifikante Unterschiede in der klonalen Zusammensetzung zwischen zufälligen und infizierten Teilen der Wirtspopulation. Dies legt nahe, dass in natürlichen Daphnienpopulationen von Parasiten verursachte Selektion wirken könnte und dass dadurch die klonale Struktur der Wirtspopulationen beeinflusst wird.

Im zweiten Teil meiner Doktorarbeit (Kapitel 5) untersuchte ich, wie Wirt-Parasit Interaktionen durch Prädation beeinflusst werden könnten. Insbesondere testete ich die potentiellen Kosten, die der Beute entstehen, wenn sie Feinden aus verschiedenen funktionalen Ebenen (d.h. Räuber und Parasiten) simultan ausgesetzt wird. Ich fand, dass der Anteil von Infektionen und die Anzahl von Parasitensporen bei den (gegen Räuber)

verteidigten Daphnien höher waren als bei den unverteidigten Daphnien. Dies zeigt zuvor unbekannte und darüber hinaus umweltrelevante Kosten induzierbarer Verteidigung auf. Diese Ergebnisse tragen dazu bei, besser zu verstehen, wie Epidemiologie in das Konzept von phänotypischer Plastizität integriert werden kann.

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GENERAL INTRODUCTION

1.1. Hybridization

A fundamental question in evolutionary biology and ecology is: which processes promote diversity of organisms in both space and time? One of such prominent processes is hybridization (e.g. Hewitt, 1988; Arnold, 1997). In contrast to relatively slow evolutionary processes, like genetic drift, mutation or gene flow, hybridization can change genetic diversity within one generation time by crossing of genetically distinguishable groups or species (i.e. interspecific hybridization, Arnold, 1997) or by exchanging genes between evolutionary lineages due to backcrossing (i.e. introgressive hybridization, Arnold, 1992). Natural hybridization and introgression was proven to be common in natural populations: at least 25% of plant species and 10% of animal species are involved in hybridization and potential introgression (reviewed by Barton and Hewitt, 1989; Arnold, 1992; Mallet, 2005).

Hybridization can result in increased genetic diversity, and form novel genotypes for the rapid adaptation to new environments (reviewed by Rieseberg and Carney, 1998). It was assumed that hybrids are more successful than parental taxa in a specific environment (i.e. “bounded hybrid superiority model”, e.g. Moore, 1977). In contrast, the “tension zone model” proposed that environments are not involved in the dynamics of hybrids; rather, the maintenance of a hybrid zone resulted from a dynamic equilibrium between natural selection against hybrids

and recurrent hybridization events on the other hand (Barton and Hewitt, 1985). The reduced fitness of hybrids, due to some pre- and postzygotic barriers between parental genomens, has been proven in several examples: the hybrids are sterile (Hewitt et al., 1987), have relatively higher embryonic mortality (Szymura and Barton, 1986), or have a lower number of offspring in comparison to parental genotypes (Keller et al., 2007). However, the role of hybridization in evolution remains debated, especially in systems where the problems of hybrid sterility (i.e. "tension zone model") can be bypassed by clonal reproduction (e.g. Lynch and Gabriel, 1990). Some theoretical studies have indicated that the rate of spread for a certain hybrid taxon relies on its relative vigour and the compatibility among hybridizing parental species (e.g. Hall et al., 2006). In the long-term, therefore, hybridization can result in the co-existence of parental species and their hybrids in most of occasions, or lead to the extinction of one or both parental species in extreme cases.

1.2. Cyclical parthenogenesis and its evolutionary significance

Cyclical parthenogenesis is a mode of reproduction combining both, sexual and asexual phases. This reproductive mode is commonly observed in nature, in both the animal and plant kingdoms (Hebert, 1987; Ellstrand et al., 1996). In cyclically parthenogenetic invertebrates, sexual and asexual generations alternate regularly in the life cycle (Simon et al., 2003). Evolutionary biologists and ecologists have intenseively studied this mode of reproduction, as it combines the advantage of sexuality with high potential of asexuality (e.g. Sunnucks et al., 1997; Taylor et al., 1999; Decaestecker et al., 2007). The best studied cyclical parthenogens are cladocerans (e.g. Hebert, 1981; Dufresne and Hebert, 1994), monogonant rotifers (e.g. Welch and Meselson, 2000) and aphids (Delmotte et al., 2003).

However, the impact of cyclically parthenogenetic reproduction, especially its asexual (clonal) component, on population structure has been largely unexplored. For instance, in the long term, clonal reproduction can decrease the effective population size by reducing the number of genetically distinct individuals (e.g. Campbell and Husband, 2005). It may also result in a spatial genetic autocorrelation, which could be falsely attributed to limited propagule dispersal or kin-structured colonization (e.g. Ingvarsson, 1998). Thirdly, as clones differ in fitness under varying environmental conditions (Vrijenhoek, 1978), changes in clone frequencies are expected across the growing season (Erickson and Hamrick, 2003). Consequently, clonal selection can result in the strong reduction of clonal diversity (Vanoverbeke and De Meester, 2010).

1.3. Hybridization in cyclical parthenogens

The hybridization (i.e. crossing between two bisexual species) is especially common in cyclically parthenogenetic systems. This phenomenon has been demonstrated in snails, crustaceans and many insects (reviewed by Simon et al., 2003). In such systems, the interspecific hybrids are produced during the short period of environmentally induced sexual reproduction, which is followed by the long period of parthenogenetic reproduction. Even if F1 hybrids have reduced sexual fertility (e.g. Barton and Hewitt, 1985), they can reach high abundances in natural populations due to clonal reproduction (e.g. Hebert, 1985; Delmotte et al., 2003). Therefore, hybrid assemblages of cyclical parthenogens are frequently composed of a mixture of individuals with clonal or sexual origins (e.g. Delmotte et al., 2003; Keller et al., 2007).

In freshwater habitats, cyclical parthenogenesis is quite common among many groups of zooplankton (Hebert, 1987). Cladocerans, which reproduce parthenogenetically during

favourable conditions and switch to sexual reproduction when conditions deteriorate (e.g. Hebert, 1978), are particularly important in these environments, as being the key component of aquatic food-webs (e.g. Edmondson and Litt, 1982; Lampert and Sommer, 1999). The cladocerans of the genus *Daphnia* (consisting of approximately 150 species, Benzie, 1988) are commonly used as a model system in evolutionary and ecological research (e.g. Matthews et al., 2011), including the fate of hybridization (reviewed in Schwenk and Spaak, 1995). When unfavourable conditions arise, such as food shortage, overcrowding or changes of temperature, *Daphnia* switch to sexual reproduction and produce males and sexual haploid eggs (Hebert, 1988). The sexual eggs need to be fertilized, which could result in a production of hybrid clones (e.g. Hebert, 1985).

Interspecific hybrids have been documented to be common within several species complexes of *Daphnia* from Eurasia, North America and Australia (Hebert, 1985; Schwenk and Spaak, 1995). However, most research has concentrated on the *Daphnia longispina* complex, which inhabits permanent lakes of the northern temperature zone (Schwenk and Spaak, 1995). In Europe, this complex is widespread, including ecologically important species *D. cucullata*, *D. galeata* and *D. longispina*, together with some rarer taxa (Petrusek et al., 2008a). These species often co-exist with their interspecific hybrids (e.g. Schwenk and Spaak, 1995; Seda et al., 2007b; Keller et al., 2008). Once *Daphnia* hybrids are produced by sexual recombination, as in other cyclical parthenogens (e.g. Ellstrand et al., 1996; Delmotte et al., 2003), they can be maintained by clonal propagation for many generations (Schwenk and Spaak, 1995), and even reach high abundances sometimes (e.g. Keller et al., 2008).

1.4. Parasite driven selection in cyclical parthenogens

Previous theoretical work has suggested that parasites can impose a strong selection pressure on their hosts, and drive the genetic changes within host populations (e.g. Jaenike, 1978; Hamilton, 1980). A key mechanism is that the host genotypes are non-randomly infected; specifically, some parasites are able to target the common host genotypes in a population (Red Queen hypothesis, Bell, 1982). Thus, if over-infected, the common host genotypes have reduced fitness. Accordingly, rare uninfected host genotypes would have a fitness advantage, and thus replace the previously common ones. At this point, the parasite genotypes would be favoured that can infect these newly common host genotypes. As a result of such non-random infection of the common versus rare host genotypes, the clonal composition of the infected part of a host population and composition of the population as a whole should be significantly different (e.g. Little and Ebert, 1999; Jokela et al., 2009). Furthermore, if infected and uninfected individuals differ in their reproductive success, this can result in selection and eventually in shifts of genotype frequencies. Such a pattern of parasite-driven negative frequency-dependant selection has been detected under laboratory conditions (e.g. Buckling and Rainey, 2002; Haag and Ebert, 2004; Lohse et al., 2006; Koskella and Lively, 2009), as well as in some natural host populations (e.g. Woolhouse et al., 2002; Decaestecker et al., 2007; Duncan and Little, 2007; Jokela et al., 2009; Wolinska and Spaak, 2009).

Natural populations of cyclical parthenogens are excellent host models to study parasite-driven selection. This is because it is possible to apply variable molecular markers to field collected samples to link the frequencies of certain host genotypes (clones) with their infection level. Moreover, we are able to follow the changes in the frequency of individual host genotypes over time during the parasite epidemic period, allowing to assess the strength of parasite-driven selection. This method has been successfully applied to populations of

cyclical parthenogens, such as freshwater snails (e.g. Dybdahl and Lively, 1998; Jokela et al., 2009) and crustacean waterflea *Daphnia* (e.g. Little and Ebert, 1999; Wolinska and Spaak, 2009).

Daphnia can be infected by a broad variety of microparasites, with fungi, bacteria, protozoa and microsporidia being particularly common (Green, 1974; Ebert, 2005). In extended field surveys, it has been shown that the parasites can infect large portions (e.g. Stirnadel and Ebert, 1997; Ebert et al., 2001; Wolinska et al., 2006; Wolinska et al., 2011) or even entire *Daphnia* populations (Lass and Ebert, 2006). Many parasite species are lethal or sterilize to their *Daphnia* hosts (e.g. Ebert et al., 2000; Hall et al., 2005; Wolinska et al., 2007a). Because of often high virulence level of these parasites, infection could be an important force shaping the *Daphnia* communities. It is not only the virulence level which is important (Howard and Lively, 1994), but also the degree of specialization (Lively, 1999). It has been shown that the *Daphnia* microparasites can represent various degrees of host specialization. For example, *Caullerya mesnili* (protozoan, class Ichthyosporea, Lohr et al., 2010b) is thought to be a specialist because it infects various host taxa and genotypes from the *D. longispina* hybrid complex to different extent (Wolinska et al., 2006; Schoebel et al., 2011). On the contrary, *Metschnikowia* sp. (yeast, family Hemiascomycetes, Wolinska et al., 2009) is believed to be a generalist. Firstly, it has been reported to infect several coexisting *Daphnia* species, which are only very distantly related: *D. magna*, *D. pulex* and *D. longispina* (Stirnadel and Ebert, 1997). In addition, identical parasite rDNA sequences were obtained from hosts, which belong to different taxa of the *D. longispina* complex (Wolinska et al., 2009).

Based on theoretical work (Howard and Lively, 1994), the high level of virulence of parasites can drive negative frequency-dependant selection in host population. Previous studies have shown the relatively quick changes in *Daphnia* host population structure that were caused by parasite infection (Duncan and Little, 2007; Wolinska and Spaak, 2009). *Daphnia* have short generation time (on average only 1-2 weeks) when compared to other model systems, which have been intensively explored in host-parasite coevolutionary studies (such as freshwater snail, e.g. Jokela et al., 2009; Koskella and Lively, 2009). So far, the most common method to track changes in host genotypes in *Daphnia* population has been allozyme electrophoresis (e.g. Little and Ebert, 1999; Duncan and Little, 2007; Wolinska and Spaak, 2009). However, only a limited number of polymorphic allozyme loci could be applied in these studies due to limited tissue availability. Therefore, a multilocus genotype as detected by a limited number of allozymes could represent a clonal group rather than a single clone. Recently, the higher resolution microsatellite markers have been successfully applied to *Daphnia* system (e.g. Thielsch et al., 2009; Pantel et al., 2011), but not yet employed in the context of parasite-driven selection.

Daphnia populations have been demonstrated to differ in infection level (e.g. Ebert et al., 2001; Hall et al., 2005; Wolinska et al., 2007a). Often, the environmental conditions have been shown to be a cause of an observed heterogeneity of infection among *Daphnia* populations (e.g. Ebert et al., 2001; Wolinska et al., 2011). For example, the food quality for host *Daphnia* could influence the parasites prevalence (Hall et al., 2009). Moreover, a recent intensive field survey (Wolinska et al., 2011) has shown that the heterogeneous pattern in parasite prevalence was observed even within single *Daphnia* populations. Therefore, it is possible to study host population structure under various degrees of parasite selection pressure,

such as different types of parasites, or the same parasite type but with different prevalence across the sites.

1.5. Host-parasite interaction affected by predation in the *D. longispina* hybrid complex

In freshwater systems, predation is an important agent of natural selection (Tollrian and Dodson, 1999). Cladocerans always have to cope with a variety of predators, such as the large, visually hunting fish and salamanders or small insect larvae with a limited gap size (e.g. Dodson, 1974; Hanazato and Ooi, 1992; Lass and Spaak, 2003; Laforsch and Tollrian, 2004). *Daphnia* are able to develop some defensive mechanisms, referred to as inducible defence, in order to increase survival. *Daphnia* are the most extensively studied system for inducible defences; many species change life-histories, such as body size and age at maturity (e.g. Spitze, 1992), and body size and number of offspring (e.g. Weider and Pijanowska, 1993). The induced behavioural defences have been observed as well. For example, *Daphnia* can migrate downward into darker and colder water layers during the daytime, where they can hide from visual predator such as fish (e.g. Ringelberg, 1991; De Meester et al., 1995; Van Gool and Ringelberg, 1995). Finally, several *Daphnia* species can respond morphologically to predators. For instance, *Daphnia pulex* can protect itself by producing neckteeth in the presence of the phantom midge larvae, *Chaoborus* (Krueger and Dodson, 1981). Other morphological structures induced by predator cues are prolongation of the tail (e.g. Mort, 1986; Black and Dodson, 1990) and building of larger helmets (e.g. Tollrian, 1990).

Many theoretical studies indicated that inducible defences could evolve when there is spatial or temporal heterogeneity in predation, and chemical cues (kairomones) can be used to indicate the risk of attack (e.g. Riessen, 1992; Hazel et al., 2004). Moreover, there must be a cost associated with the defended type because of the evolutionary stability of inducible

defences; otherwise, we would expect the defence to be constitutively expressed (e.g. Vantienderen, 1991; Riessen, 1992). It has been suggested that the reduction in growth and development could be the cost of inducible defences (e.g. Clark and Harvell, 1992). For example, for the *Daphnia* system, induced morphs (with neckteeth), suffer a 11% to 39% lower population growth rate than undefended ones (e.g. Black and Dodson, 1990). In addition, the time to first reproduction for defended *Daphnia* is about 5-15% longer than in undefended morphs (e.g. Black and Dodson, 1990). However, this observed type of costs (in growth and development) has been questioned. It has been argued that the increased time to maturity may be not a direct physiological cost of the morphologically defended type, but a trade-off for its larger body size (Tollrian, 1995). Therefore, it is uncertain if commonly observed demographic changes (e.g. Black and Dodson, 1990) can represent costs in the formation of morphological defences, or just represent trade-offs for the induced changes of life-history parameters.

Indirect cost has been suggested to be an alternative explanation for the evolution of inducible defences (Tollrian and Harvell, 1999). Specifically, different hunting strategies can result in mismatches between induced defences and predation risk. For example, smaller *Daphnia* are advantageous because fish feed selectively on larger prey (Spaak and Hoekstra, 1997). However, smaller *Daphnia* are at disadvantage when they encounter the invertebrate predators, which preferentially attack smaller prey (Riessen, 1999). Therefore, in the presence of fish, *Daphnia* mature at an earlier age (at smaller size) and produce more but smaller offspring in order to avoid detection by visually hunting predators (e.g. Boersma et al., 1998). When invertebrate predators are the most dominant threat, however, *Daphnia* will mature later and produce a clutch of larger offspring (e.g. Stibor and Lüning, 1994). Consequently, as the cost of mismatched defences can be large, the fluctuating presence of predators with

contrasting hunting strategies could promote the evolution of inducible traits (Taylor and Hebert, 1993). Although numerous studies have explored the costs in a context of two contrasting hunting strategies (reviewed by Tollrian and Harvell, 1999), the potential costs of simultaneous prey exposure to enemies from different functional levels, such as predators and parasites, remain unexplored. Therefore, host-parasite interactions in the *D. longispina* hybrid complex could be also explored in a context of predation.

1.6. Outline of this thesis

The aim of this PhD project was to extend our understanding of population structure and host-parasite interactions in the *D. longispina* hybrid complex. First, I needed to test how the high number of microsatellite markers would enhance the partitioning of genotypic variation in the studied here *D. longispina* hybrid complex. Because of the low discriminatory power of a small number of nuclear allozyme markers, which mostly have been used for *Daphnia* in the past (e.g. Taylor and Hebert, 1992; Gießler, 1997b), the information may be insufficient to identify transient species boundaries resulting from introgression in a hybrid complex. Thus, in Chapter 2, I first calibrated the species assignment by microsatellite markers using the association between different sets of nuclear genetic markers (such as previously used allozymes and recently established microsatellite markers) in reference clones. Secondly I used different statistical approaches (Factorial Correspondence Analysis, NewHybrids and STRUCTURE) to assess the accuracy of individual assignments to different hybrid classes in my field samples. Then, in Chapters 2 and 3, I tested the hypothesis that the genotypic diversity should be lower in hybrids than in parental species, because there are some pre- and postzygotic barriers between parental genomes (e.g. Keller et al., 2007), which can result in a lower number of newly produced hybrid in comparison to parental genotypes. In order to assess the impacts of clonal reproduction on community structure in cyclical parthenogens,

Chapter 3 addresses dynamics in taxon and clonal structure, across time (generation-to-generation) and space (between sampling stations), during a period of seasonal environmental change.

In the following two chapters (i.e. 4 and 5), I studied parasitism in hybridizing *D. longispina* systems. In Chapter 4, I investigated parasite-mediated selection in host populations. Thus, I compared the clonal composition of the infected part of a host population and the composition of the population as a whole. Furthermore, I studied parasite-driven selection in host populations under the presence of two different parasites. In Chapter 5, I investigated to what extend host-parasite interactions are altered by signals from a predator (i.e. kairomones). Specifically, I tested the prediction that the predator-induced individuals would show greater infection prevalence and intensity than the individuals not exposed to kairomones, which would result in significant costs of inducible defences.

The thesis concludes with Chapter 6, providing a general discussion and suggestions for future research.

CLONAL DIVERSITY, CLONAL PERSISTENCE AND RAPID TAXON REPLACEMENT IN NATURAL POPULATIONS OF SPECIES AND HYBRIDS OF THE *DAPHNIA LONGISPINA* COMPLEX

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Clonal diversity, clonal persistence and rapid taxon replacement in natural populations of species and hybrids of the *Daphnia longispina* complex

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Abstract

Hybridization is common among cyclical parthenogens, especially in zooplankton species assemblages of the genus *Daphnia*. To explore hybridization dynamics and the extent of clonal diversity in the *Daphnia longispina* complex, we analysed population structure in eight permanent lakes. Based on 15 microsatellite loci, three major taxonomic units emerged: two species, *D. galeata* and *D. longispina* and their F1 hybrids, supported by factorial correspondence analysis and two Bayesian methods. At the same time, the detection of backcross classes differed between methods. Mean clonal diversity was lowest in the F1 hybrids, as expected from the high rate of asexual reproduction. Within taxa, replicated genotypes were of clonal origin, but clonal lineages persisted in subsequent years in only one of three resampled lakes. In another lake, the taxon composition changed from being dominated by hybrids to complete dominance by one parental taxon. Such a year-to-year taxon replacement has not been reported for the *D. longispina* complex before. Our data on this hybrid complex illustrate that high-resolution genotyping is essential for the understanding of ecological and evolutionary outcomes of hybridization in partially clonal taxa.

Keywords: cyclical parthenogenesis, *Daphnia longispina complex*, genetic diversity, microsatellite, population structure

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Introduction

Hybridization, the interbreeding of species, is common in natural populations. At least 25% of plant species and 10% of animal species are involved in hybridization and potential introgression with other species (Mallet 2005). Despite this, the role of hybridization in evolution is still debated, especially in systems where the problems of hybrid sterility can be bypassed by clonal reproduction (although this bypass bears the cost of reduced clonal diversity via Muller's ratchet and natural selection, Lynch & Gabriel 1990). Theoretical studies have shown that the rate at which hybrid taxa spread depends on their relative vigour and the compatibility among hybridizing parental species (Hall *et al.* 2006). In the long-term, hybridization can result in the coexistence of parental species and their hybrids, or in the extinction of one or both species in extreme cases. It may also lead to the merging of hybridizing gene pools or the reinforcement of reproductive barriers through selection (Arnold 1992).

Before conclusions about the significance of hybridization can be drawn, one major challenge is the fine-scale partitioning of genotypic variance into taxonomic units. The accurate classification of individuals in complex systems encompassing many generations of hybridization and backcrossing requires a large set of molecular markers to disentangle taxonomic units (Boecklen & Howard 1997). Fortunately, various molecular markers (e.g. Mort & Wolf 1986; Brede *et al.* 2006), population genetic tools and Bayesian approaches (e.g. Pritchard *et al.* 2000; Anderson & Thompson 2002; Halkett *et al.* 2005) are now available to resolve

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genotypic identity within hybridizing taxa. This is important because species designation is of high ecological relevance and is a prerequisite to understanding the role and persistence of hybrids in an evolutionary context.

In animals, hybridization is especially common among groups that reproduce by cyclical parthenogenesis, for example among zooplanktonic cladocerans (Hebert 1985), or in some insects (Delmotte *et al.* 2003). In such systems, long periods of parthenogenetic reproduction alternate with short periods of environmentally induced sexual reproduction where interspecific hybrids are produced. Even if F1 hybrids have reduced sexual fertility, they may reach high abundances in natural populations because of parthenogenetic (clonal) propagation (Hebert 1985; Delmotte *et al.* 2003; Keller *et al.* 2007). Thus, hybrid assemblages of cyclical parthenogens are frequently composed of a mixture of individuals with clonal or sexual origins. Many hybrid complexes have been identified in cladocerans of the genus *Daphnia*, mostly using allozymes as codominant nuclear markers (Taylor & Hebert 1992; Gießler 1997a). In these systems, hybrids are involved in backcrossing and introgression (e.g. Hebert *et al.* 1989; Schwenk & Spaak 1995; Gießler 1997a; Keller *et al.* 2007). When species boundaries are transient because of introgression, the discriminatory power of a small number of nuclear loci may be insufficient to identify taxonomic units (Boecklen & Howard 1997). Thus, supposedly diagnostic nuclear markers, such as the ribosomal internal transcribed spacer (ITS) (Billiones *et al.* 2004; Taylor *et al.* 2005; Skage *et al.* 2007; Petrusek *et al.* 2008b) can give the wrong signals for delineating taxa in hybridizing *Daphnia* assemblages. This result is consistent with the finding of reticulate patterns for some alleles in the *Daphnia longispina* complex (Gießler & Englbrecht 2009). Thus, multi-locus genotyping at polymorphic nuclear loci is required to discriminate among parental species, different hybrid classes and nuclear introgression.

Microsatellites are the most powerful markers available to date for tracing clonal lineages. Only recently, microsatellite markers have been developed for hybridizing *Daphnia* (i.e. for the *D. longispina* hybrid complex, Brede *et al.* 2006). However, little is known about the genetic structure of hybrid groups in this species complex. The few studies that have employed these markers have focused on the composition of egg banks (Brede *et al.* 2009), the association between presumed species-specific markers (Dlouhá *et al.* 2010) or the population structure of one parental species (Thielsch *et al.* 2009). The latter study using 13 microsatellite loci showed that, in most populations, all genotyped specimens of parental *D. longispina* were genetically distinct (Thielsch *et al.* 2009). This is unexpected, as daphnids reproduce clonally during most of the year (Hebert 1985; Keller *et al.* 2007) and genotypes favoured by natural selection are presumed to be more abundant than others (Vrijenhoek 1979). Furthermore, no genotype was shared among the lakes, suggesting that gene flow and migration are negligible (Thielsch *et al.* 2009). If the recent finding of surprisingly high genotypic diversity can be generalized for all taxa within the hybridizing *D. longispina* complex, previous estimates of clonal diversity derived from a few allozyme loci were far too low (e.g. Mort & Wolf 1985; Spaak 1996). Corresponding mismatches between estimates derived from allozymes and microsatellites have been reported from other hybridizing systems, such as aphids (Delmotte *et al.* 2002). Thus far, data are limited regarding the clonal structure and population dynamics when high-resolution genotyping is applied in natural hybrid assemblages; new conclusions may emerge when a high number of polymorphic loci are used for the discrimination of taxa and clonal lineages.

In this study, we used 15 microsatellite loci to genotype individuals of the *D. longispina* complex, originating from eight lakes. First, we wanted to know how the high number of microsatellite markers would enhance the partitioning of genotypic variation in this partially clonal hybrid complex. Therefore, we applied different statistical approaches to assign species and additionally looked for the diagnostic alleles. Then, we tested whether the recently uncovered high genotypic diversity of one parental species (Thielsch *et al.* 2009) holds for other taxa in the *D. longispina* complex. Specifically, we hypothesized that the genotypic diversity should be lower in hybrids, as they mostly reproduce parthenogenetically (Hebert 1985; Keller *et al.* 2007) and are thus present as clonal lineages. Moreover, Thielsch *et al.* (2009) detected no genetic isolation by distance; their study populations, however, were separated by hundreds of kilometres. We tested whether a genetic isolation by distance emerges at a smaller geographical scale. Finally, we examined temporal variation in taxon and clonal composition, by re-sampling three of the eight studied lakes in the following year, after an expected period of sexual reproduction.

Materials and methods

Daphnia collection from natural populations

The study was carried out in four small quarry lakes in Munich (Germany) and in four natural lakes near the city (Table 1). The lakes were sampled for zooplankton in late spring or early autumn in 2008. In addition, three of the lakes were re-sampled in spring 2009. Samples were taken with a 95-μm zooplankton net hauled through the whole water column at two to three

Table 1 Genetic diversity of *Daphnia* populations based on 15 microsatellite loci

Lake (abbreviation)	Latitude, longitude	Surface area (km^2)	Origin	Sampling period[a]	Taxon[b]	N[c]	N[d]	N_a	H_o	H_e	HWE	F_{IS}	MLG	MLG/N[d]	E_{var}
Ammersee (AMME)	48.034N, 11.702E	46.60	Natural	Autumn	*Daphnia longispina*	5	5	50	0.53	0.54	0.58	0.03	5	1.00	1.00
					D. galeata	12	12	37	0.56	0.45	*	−0.25	11	0.92	0.97
					F1 hybrids	61	60	64	0.86	0.60	**	−0.44	28	0.47	0.72
Feldmochinger See (FELD)	48.125N, 11.305E	0.16	Artificial	Spring	F1 hybrids	44	41	33	0.88	0.47	***	−0.92	7	0.17	0.39
				Spring^{2009}	*D. galeata*	46	41	45	0.48	0.38	***	−0.27	26	0.63	0.82
Feringasee (FERI)	48.114N, 11.401E	0.32	Artificial	Spring	*D. galeata*	31	29	45	0.56	0.51	0.06	−0.09	29	1.00	1.00
				Spring^{2009}	*D. galeata*	37	37	60	0.47	0.45	0.83	−0.03	37	1.00	1.00
Heimstettener See (HEIM)	48.092N, 11.441E	0.11	Artificial	Spring	*D. galeata*	46	43	56	0.45	0.44	0.94	−0.04	43	1.00	1.00
Pilsensee (PILS)	48.013N, 11.113E	1.95	Natural	Autumn	*D. longispina*	43	39	63	0.40	0.44	1.00	0.09	31	0.80	0.93
Starnberger See (STAR)	47.543N, 11.184E	56.36	Natural	Autumn	F1 hybrids	39	37	63	0.81	0.57	***	−0.44	25	0.68	0.89
Waldschwaig See (WALD)	48.132N, 11.261E	0.11	Artificial	Spring	*D. longispina*	86	79	62	0.47	0.39	0.93	−0.21	42	0.53	0.76
				Spring^{2009}	*D. longispina*	46	34	43	0.35	0.35	0.34	−0.03	22	0.65	0.83
Wesslinger See (WESS)	48.042N, 11.151E	0.17	Natural	Autumn	F1 hybrids	42	42	70	0.82	0.61	***	−0.36	29	0.69	0.88

[a]The samples were taken in 2008, if not indicated otherwise.
[b]The taxon membership was defined by the NewHybrids software based on the allelic variation at 15 microsatellite loci (only few individuals were classified to the backcross taxa, and those are not included here).
[c]Total number of individuals.
[d]Number of individuals excluding the missing data; *N*a, number of alleles; *H*o, observed heterozygosity; *He*, expected heterozygosity; HWE, Hardy-Weinberg-Equilibrium; FIS, inbreeding coefficient; MLG, number of unique multi-locus genotypes; *E*var, evenness; ***$P < 0.001$;**$P < 0.01$;*$P < 0.05$.

different sites within the deep basin. For each lake, samples from different sites were mixed. From each mixed sample, randomly selected adult females from the *Daphnia longispina* complex were individually frozen at −80 °C or preserved in 95% ethanol for genotyping. In total, 441 *Daphnia* were analysed from eight lakes sampled in 2008 and 135 from three lakes re-sampled in 2009.

Reference taxa

Altogether 49 well-defined genotypes from the *D. longispina* complex were used as a reference to verify the correspondence of taxon membership derived from different genetic markers. They were sampled between 1985 and 2007 across 27 locations in Europe and one location in North America. All animals were maintained thereafter in the laboratory as clonal lineages (for sampling locations see Table S1, Supporting information). Daphnids were first identified by morphology according to Benzie (2005) as *D. cucullata*, *D. galeata*, *D. hyalina*, *D. rosea* and their hybrids. Relying on a recent taxonomic revision, we do not discriminate here between the two morphs '*hyalina*' and '*rosea*', which were previously considered distinct species but are now pooled as '*D. longispina*' (Petrusek *et al*. 2008a). In addition, all individuals were genotyped based on allozyme loci, recognized to be diagnostic for species identification (Gießler 1997a). Finally, the associated mtDNA type was analysed by RFLP of cytochrome b (Gießler *et al*. 1999) and/or by the sequence information of the 12S region (Gießler & Englbrecht 2009).

DNA extraction and microsatellite genotyping

In total, 625 *Daphnia* (49 reference genotypes and 576 field collected individuals) were individually incubated in 60-100μL of H3 buffer with proteinase K, containing 10 mM Tris–HCl, 0.05 M potassium chloride, 0.005% Tween 20, 0.005% NP-40 and 1.5 μg/mL proteinase K (Sigma), for 10–16 h at 55 °C. Then, samples were heated to 95 °C for 12 min to inactivate the proteinase K, centrifuged shortly and stored at 4 °C. Fifteen previously published microsatellite markers (Brede *et al*. 2006) were used in two sets of multiplex polymerase chain reactions (MP1: Dgm105, Dgm112, SwiD5, SwiD7, SwiD8; and MP2: Dgm109, Dp196, Dp281, Dp512, SwiD1, SwiD2, SwiD10, SwiD12, SwiD14, SwiD15), using the Qiagen Multiplex PCR Kit. The forward primers were labelled with the fluorescent dyes: 6-FAM (Dp196, Dp281, SwiD8, SwiD10), Vic (Dgm105, Dgm109, Dgm112, Dp512, SwiD2, SwiD7, SwiD15), PET (SwiD5, SwiD12, SwiD14) and NED (SwiD1) (Applied

Biosystems). The concentration of each primer was adjusted to optimize scoring of the fluorescent peaks. The amplification protocol consisted of the initial step of 15 min at 95 °C to activate the DNA polymerase and denature the template DNA, followed by 33 cycles of 30 s at 94 °C, 1 min 30 s at 56 °C (MP1) or 54 °C (MP2), 1 min at 72 °C and a final extension step of 30 min at 60 °C. PCR products were analysed on an ABI 3700 capillary sequencer using a LIZ 500 labelled size standard. Genotypes were read using GeneMapper 3.7 software (Applied Biosystems). Alleles at each locus were defined based on the base-pair length of the fragments.

Statistical analyses

Preparing the data set. Before the data sets from different runs were merged, the consistency of alleles was checked against locus-specific patterns for two reference genotypes used in each run. This enabled us to adjust for alleles with small differences in fragment length. There was no evidence for scoring errors because of stuttering, large allele dropout or presence of null alleles, as indicated by respective tests using MICRO-CHECKER 2.2.3 (10^4 permutations, van Oosterhout *et al*. 2004).

Taxon assignment and composition. To display the genetic relatedness of individuals which were genotyped at 15 microsatellite loci, factorial correspondence analysis (FCA) was applied (GENETIX 4.05, Belkhir *et al.* 1996-2004). The clusters that emerged in FCA were analysed further for phylogenetic relationships based on their associated 12S mtDNA genotypes (fragment length: 252 bp). The phylogenetic analysis was restricted to a subset of reference clones. A neighbour-joining tree was generated in MEGA 4 (Tamura *et al.* 2007) (complete deletion option, Kimura 2-parameter model, 1000 × bootstrap). For field samples, we additionally applied two model-based Bayesian methods. In NewHybrids 1.1 (Anderson & Thompson 2002), the individuals were assigned to one of six predefined taxa (two parental and four hybrid groups: F1, F2 and both backcrosses) applying a threshold of 95% posterior probability (10^6 iterations after a burn-in of length 10^6). In STRUCTURE 2.2 (Pritchard *et al.* 2000), the most likely number of taxonomic units was estimated from the same data set by running the algorithm with values of K from 1 to 15 using the admixture model (Evanno *et al.* 2005) and 10^5 iterations after a burn-in of length 10^6. Finally, we compared the assignment of individuals as determined by the two Bayesian approaches (using an R × C test, Sokal & Rohlf 1995). All the following calculations of taxon-specific parameters are based on the taxon assignment in NewHybrids.

Allelic diversity. Per locus and population, allelic richness (A_r) was computed in FSTAT 2.9.3 (Goudet 1995). Private and thus potentially taxon-specific alleles (i.e. the alleles which are only present in a certain taxon) were identified in CONVERT (Glaubitz 2004), using the 49 reference genotypes and the 441 individuals sampled in 2008. The abundance of private alleles per locus (A_P) was calculated as the ratio of $\sum A_S$ (sum of private alleles) and $\sum A_T$ (sum of all alleles). If the abundance of private alleles (A_P) was greater than 5% for both parental taxa, then the respective loci were assumed to be useful for taxa discrimination.

Genetic diversity. For each population, genetic diversity was examined by calculating the number of alleles (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e), using GENALEX 6 (Peakall & Smouse 2006). Exact tests for significant deviation from Hardy-Weinberg equilibrium (HWE) were computed in GENEPOP 3.4 (10^4 permutations, Raymond & Rousset 1995). The inbreeding coefficient (F_{IS}), which measures the extent of nonrandom mating, was calculated in Arlequin 3.0 (10^4 permutations, Excoffier *et al*. 2005). Unique multilocus genotypes (MLGs) were identified using GENCLONE 2.0 (Haond & Belkhir 2007), where individuals with missing data (at one or more loci) were excluded. The probabilities that individuals possessing identical MLGs resulting from sexual recombination (P_{sex} values) were calculated using MLGsim (Stenberg *et al.* 2003). To evaluate the distribution of MLGs in each population, the evenness index E_{var} (Smith & Wilson 1996) was calculated.

Spatial variation. We applied an analysis of molecular variance (AMOVA) to partition the genotypic variance into within-population and among-population components (10^4 permutations). Furthermore, to estimate genetic differentiation among conspecific *Daphnia* populations originating from different locations, the fixation index F_{ST} was calculated in Arlequin 3.0 (10^4 permutations). The correlation between pairwise geographical distances and pairwise F_{ST} was evaluated using a Mantel test (10^4 permutations).

Results

Taxon assignment and composition

The 49 reference genotypes covered three parental (*Daphnia cucullata*, *D. galeata*, *D. longispina*) and two hybrid taxa (F1 *D. cucullata* × *D. galeata* and F1 *D. galeata* × *D. longispina*) as defined by allozymes (Table S1, Supporting information). Based on allelic variation at 15 microsatellite loci, three main clusters of MLGs and

some intermediates were revealed by FCA analysis (Fig. 1a). The correspondence of taxon identification by allozymes and microsatellite markers was 98%; only one genotype was assigned differently by the two methods (genotype O2; Table S1, Supporting information). The identification of taxa was 100% supported by respective mtDNA genotypes (Table S1, Supporting information). Well-resolved clusters of three parental taxa in the mtDNA neighbour-joining tree (inset in Fig. 1a) mirrored the clusters in the FCA plot.

To infer population structure in samples from eight lakes, we applied three different methods using the information of allelic variation at 15 microsatellite loci. According to FCA, the MLGs of 441 field collected *Daphnia* in 2008 clustered around the reference taxa: *D. galeata*, *D. longispina* and the respective hybrids (Fig 1a, b). In the NewHybrids analysis, the MLGs were assigned to five taxonomic groups (two parental and three hybrid groups: F1, and both backcrosses); only 17 individuals could not be assigned to any of these groups by the 95% posterior probability. Four lakes were dominated by F1 hybrids, two by *D. longispina* and two other by *D. galeata*. In most lakes, the abundance of backcross classes was <10%. Only Feringasee had more backcrossed individuals (~15%) and only Ammersee had four different taxa which co-occurred (Fig. 2). There was good correspondence in taxon assignments between NewHybrids and STRUCTURE. Specifically, 91% of *D. galeata* and 90% of *D. longispina* were assigned to the corresponding unit by STRUCTURE (Fig. 3). Remarkably, the F1 hybrid group, as identified by NewHybrids, was split into three subgroups by STRUCTURE while the backcross classes remained unidentified. One subgroup consisted of two F1 hybrid populations (originating from Ammersee and Starnberger See), whereas the other two subgroups comprised a single F1 population each from Wesslinger See and Feldmochinger See, respectively. Finally, we used the NewHybrids assignment to

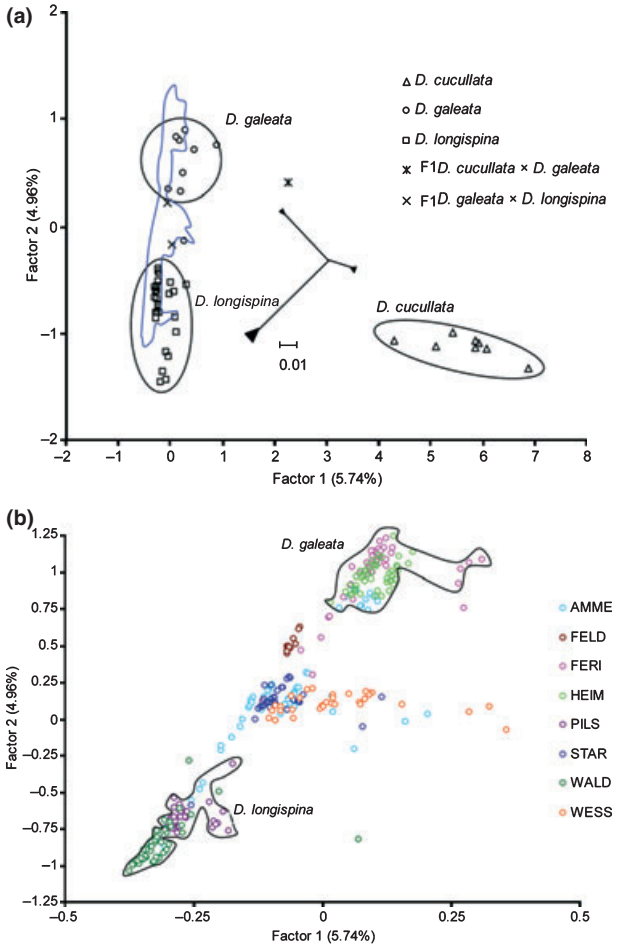

Fig. 1 Factorial correspondence analysis based on allelic variation at 15 microsatellite loci. FCA scores at the first two axes are shown for (a) 49 reference genotypes representing three parental taxa (and, additionally, three hybrid genotypes) of the European *Daphnia longispina* complex, as defined by allozyme markers (see Table S1, Supporting information). Enclosed with a blue outline are FCA coordinates for the field samples as magnified in (b). The neighbour-joining tree in the middle of the plot displays three species-specific lineages based on 12S mtDNA sequence variation, supported by bootstrap values close to 100%; scale bar represents units of divergence (12S data from Gießler & Englbrecht 2009 are supplemented with additional data from reference clones; data will be published elsewhere). (b) In 2008, 441 individuals sampled from eight lakes, the two outlines enclose the two parental taxa according to the assignment by NewHybrids. Note the scale change. For abbreviations of lakes see Table 1.

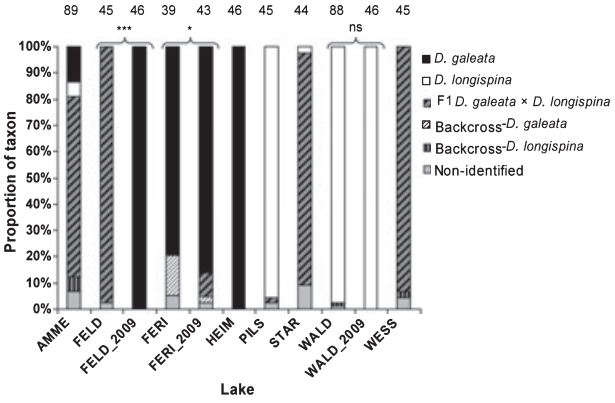

Fig. 2 *Daphnia* taxon composition of eight lakes sampled in 2008 and, for three of these lakes, the re-sampling in 2009 (based on allelic variation at 15 microsatellite loci and NewHybrids assignment). Significant differences between years are denoted as $*P < 0.05$, $***P < 0.001$ (ns – not significant). Sample sizes are given at the top of each bar. For lake abbreviations see Table 1.

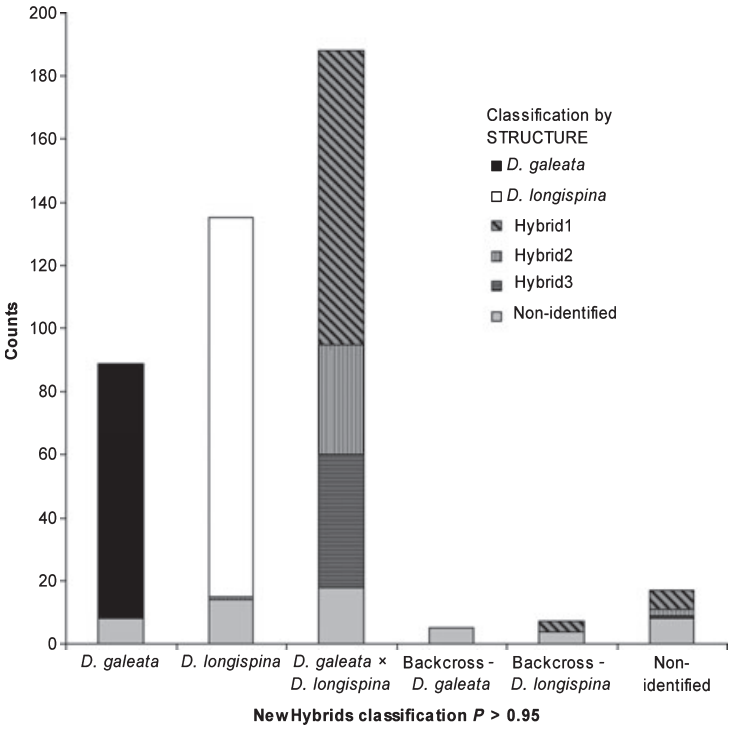

Fig. 3 Correspondence between NewHybrids and STRUCTURE (K = 5) group assignments for 441 individuals from eight lakes based on allelic variation at 15 microsatellite loci. The F1 hybrid taxon (as classified in NewHybrids) is split into three subgroups by STRUCTURE.

parental and F1 taxa for the calculation of population parameters.

Allelic diversity

At 15 microsatellite loci, we detected 244 different alleles from a total of 49 reference genotypes and 441 individuals sampled in 2008. We scored 104 alleles in 97 *D. galeata* individuals, 158 alleles in 160 *D. longispina* individuals and 118 alleles in 186 screened F1 hybrids (15 alleles present in F1 hybrids were not detected in any parental taxon). The mean allelic richness per locus and population (A_r) was larger in hybrids than in parental (t = 2.1, $P < 0.05$; Table S3, Supporting information). The proportion of private alleles (A_P) was high: 41% in *D. galeata* and 61% in *D. longispina*. Five loci had private allele abundances >5% for both parental taxa (i.e. SwiD5, SwiD7, SwiD10, SwiD12, and SwiD15) and are thus recommended for species discrimination (Table S3, Supporting information).

Genetic diversity

Among 499 individuals with complete MLG profiles (2008 and 2009 samples), 335 unique MLGs were detected. Individuals possessing identical MLGs can be regarded as true clones; in all populations P_{sex} values were lower than 10^{-6} ($P < 0.05$), rejecting the hypothesis that identical MLGs were of sexual origin. The ratio of distinct genotypes (i.e. MLG/N) was higher in parental than in F1 hybrids; t = 2.7, $P < 0.05$ (the difference remained significant after excluding two populations with sample size <15 or the other three populations that were collected in 2009, see Table 1). Specifically, in four of nine screened parental populations, each genotyped *Daphnia* had a distinct MLG (i.e. MLG/N = 1.00), and this was not the case in any of the four screened F1 hybrid populations (Table 1). Conversely, in one of the F1 hybrid populations (Feldmochinger See), the MLG/N ratio was only 0.17. Additionally, the distribution of genotypes was highly uneven (E_{var} = 0.39) in this population (index of 1.00 would otherwise indicate that all clones were present in equal frequencies). All four F1 hybrid populations and two of nine parental populations showed strong deviations from Hardy-Weinberg equilibrium. In all these populations, negative values of F_{IS} (Table 1) indicated an excess of heterozygotes. Mean F_{IS} was significantly more negative in F1 hybrids than in parental taxa (t = 3.2, $P < 0.05$).

Spatial variation

AMOVA indicated that the within-population variance explained most of the genetic diversity (from 73% to 84%), but there was also significant variance among populations (Table 2). Pairwise F_{ST} values among populations ranged from 0.05 to 0.38 (averaged over all loci). The graphical display suggested genetic isolation

Table 2 Hierarchical analysis of molecular variance (AMOVA) among populations and within populations (calculated per *Daphnia* taxon). Only samples from 2008 were included

Taxon	Source of variation	d.f.	Sum of squares	Percentage of variation	*P* value
Daphnia galeata	Among-populations	2	70.99	16.00	<0.001
	Within-populations	175	561.61	84.00	<0.001
Fl hybrids	Among-populations	3	423.21	26.95	<0.001
	Within-populations	368	1486.25	73.05	<0.001
D. longispina	Among-populations	2	86.14	18.66	<0.001
	Within-populations	265	719.21	81.34	<0.001

by distance for two taxa (*D. longispina* and F1 hybrids, Fig. 4). Although the correlation was high for F1 hybrid populations (R^2 = 0.69), the relationship was not significant (P = 0.21) because of the small sample size (i.e. only four populations). The test was not computed for *D. longispina* because only three populations were sampled. In contrast, there was no evidence of isolation by distance for *D. galeata* (R^2 = 0.01, P = 0.45; four populations).

Temporal variation

Between 2008 and 2009, the taxonomic composition of *Daphnia* assemblages changed in two of three re-sampled lakes. The spatial and temporal patterns are shown in Fig. 5. In Feldmochinger See, the change was dramatic: from complete dominance by the F1 hybrids,

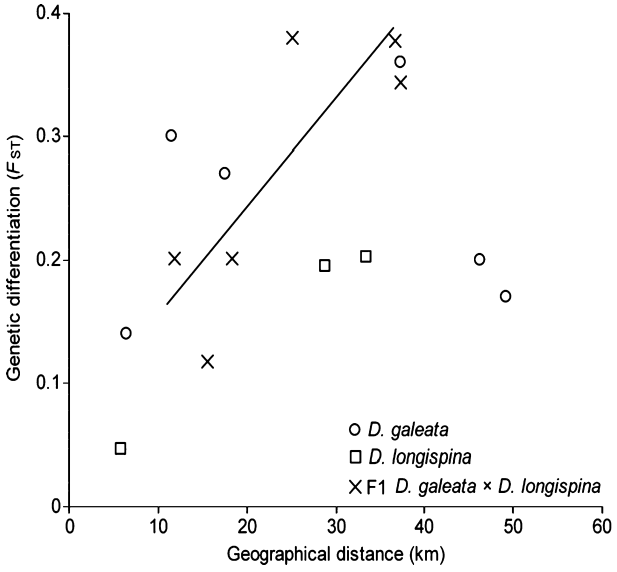

Fig. 4 Scatterplot of pairwise geographical distances (kilometres) versus genetic distance (F_{ST} based on 15 microsatellite loci) among conspecific populations of the *Daphnia longispina* complex. The regression line is drawn for populations of F1 hybrids (the correlation was high: R^2 = 0.69).

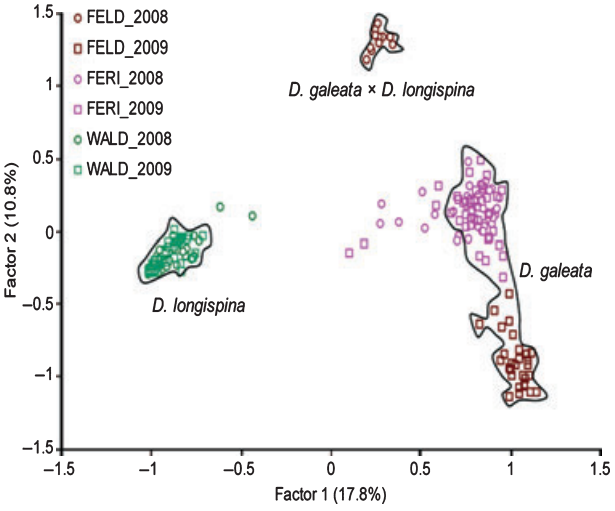

Fig. 5 Factorial correspondence analysis based on allelic variation at 15 microsatellite loci. The *Daphnia* populations from three lakes, which were sampled in both 2008 and 2009 (see Fig. 2), are shown in details. Each lake is indicated by a different colour. The 2009 samples are shown in squares. For lake abbreviations see Table 1.

to a complete dominance of *D. galeata* (Figs 2 and 5). There were no MLGs maintained across the 2 years, except in the *D. longispina* population from Waldschwaig See: five of 42 MLGs detected in 2008 were also present in 2009. Two of the five shared MLGs were the most frequent ones in both years, representing 16% and 11% of all individuals in the 2008 sample, compared to 12% each in the 2009 sample.

Discussion

Interspecies and intraspecies population dynamics in partially clonal hybrid complexes can now be traced by high-resolution genotyping. Here, we applied a larger set of microsatellite markers than ever before to identify genotypes in the taxonomically problematic *Daphnia longispina* hybrid complex. Based on this set of markers, we present data on the clonal diversity and the spatial

and temporal genetic population structure of hybrid and parental taxa.

To corroborate the taxonomic assignment of individuals from field samples, which was based purely on microsatellites, we used reference taxa genotyped by allozymes, mtDNA and microsatellite markers. There was a nearly perfect correspondence in assignments using the 15 microsatellites and other markers, similar to recent findings for *Daphnia* individuals from other geographical regions with a different selection of microsatellites (Thielsch *et al.* 2009; Dlouhá *et al.* 2010). Furthermore, field collected *Daphnia* were consistently assigned to major taxonomic groups by three independent statistical approaches (FCA and two Bayesian methods). In both analyses, NewHybrids and STRUCTURE, five taxonomic units were detected but only two of them, representing the parental species, were consistent. Using STRUCTURE, the F1 hybrid group, as assigned by NewHybrids, was further split into three subgroups associated with lake of origin. Two subgroups fully corresponded to respective F1 populations from two lakes, suggesting local selection as well as local production of hybrids and that STRUCTURE is sensitive to detect even small differences within taxonomic units. The third subgroup combined two F1 populations from Ammersee and Starnberger See; these two habitats are large natural lakes, possibly providing similar selection pressure on hybrid populations. On the other hand, the two backcross classes from NewHybrids were not identified by STRUCTURE. One explanation for this discrepancy is that accurate detection of the F2 and backcross classes requires more loci (Vaha & Primmer 2006). The mismatch may also result from the fact that STRUCTURE is not designed for organisms which reproduce mainly asexually (Pritchard *et al.* 2000; Falush *et al.* 2003; Halkett *et al.* 2005). Regardless of this discrepancy, we were able to identify some species-diagnostic microsatellite alleles by applying statistical criteria for this large data set. Five of the 15 microsatellites tested here may serve in future studies to identify the species *D. galeata* and *D. longispina*.

Rates of clonal reproduction have a large impact on the population structure. For example, the effective population size is a function of clonal diversity (Halkett *et al.* 2005). Therefore, we compared evidence for clonal propagation in F1 hybrid and parental taxa. In agreement with a recent microsatellite study of one parental taxon from the same species complex (i.e. *D. longispina* in Thielsch *et al.* 2009), the observed genetic diversity in all taxa examined was much higher than previously determined by allozymes (Mort & Wolf 1985; Spaak 1996; Gießler 1997b). This difference can be explained by the larger number of loci screened, combined with higher polymorphism at single loci for microsatellites (Arnaud-Haond *et al.* 2007). Nevertheless, the F1 hybrids had, on average, lower diversity than parental taxa, as expected by predominantly clonal reproduction in hybrid populations (Keller *et al.* 2007). Individuals with identical MLGs were indeed shown to belong to clonal lineages. A similar pattern of increased asexual reproduction in hybrids is often observed in plant species, leading in turn to the long-term maintenance of hybrid populations (Ellstrand *et al.* 1996). Moreover, as predicted for populations with primarily clonal reproduction (Balloux *et al.* 2003), allelic richness was higher in F1 hybrids than in parental taxa. Similar difference in allelic richness has recently been detected between asexual and sexual populations of aphids (Kanbe & Akimoto 2009). Furthermore, all four hybrid populations showed strong deviations from Hardy-Weinberg equilibrium, compared to only two of nine investigated parental taxa populations. This was correlated with an excess of heterozygotes in the hybrids, arguably caused by asexual reproduction (see Delmotte *et al.* 2002).

Across the studied lakes, we found two parental taxa, F1 hybrids and two backcross classes (as defined by NewHybrids). All taxa coexisted in various combinations and proportions as previously observed using allozyme markers in other populations of the *D. longispina* complex (e.g. Keller *et al.* 2008; Petrusek *et al.* 2008a). In most of the lakes studied here, however, only one or two taxa were abundant. Only in Ammersee, which was one of the two largest lakes in our study, four different taxa were present simultaneously. This is consistent with the expectation of enhanced genetic diversity in larger habitats (Michels *et al.* 2003), because of an increasing number of ecological niches, allowing the coexistence of ecologically different genotypes (De Meester 1996). Moreover, larger lakes should have a greater stock of resting eggs and hence a higher total recruitment of new, sexually produced genotypes (Korpelainen 1986) which should further enhance genetic diversity.

The F_{ST} values found ranged from 0.05 to 0.38, indicating low to high genetic differentiation among populations from each taxon and corresponding with some previous small-scale *Daphnia* field surveys (Gießler 1997a; Michels *et al.* 2003). These values are lower than values observed by Thielsch *et al.* (2009), although both estimates were based on a similar number of microsatellite markers (i.e. 13 loci in Thielsch *et al.* and 15 loci in our study). Because it is predicted that the larger an investigated region, the higher genetic differentiation will be observed (Slatkin 1985), one likely explanation is the different spatial scale between the studies. The longest distance between lakes in our study was only ~49 km, compared to ~3500 km in Thielsch *et al.* (2009). Nevertheless, we found significant among-population variance. Identical multilocus genotypes were not

shared among populations which would be direct evidence of gene flow. However, there was a trend of increasing genetic differentiation with increasing geographical distance for F1 hybrids ($R^2 = 0.69$, Fig. 4). Though, unfortunately, the power of the Mantel test was too low to corroborate this trend. However, the observed trend is consistent with a previously reported significant association between genetic distance (measured by allozyme patterns) and geographical distance over a small-scale for taxa of the *D. longispina* complex, including hybrids (Gießler 1997b). In contrast, a recent large-scale study of *D. longispina* (Thielsch *et al.* 2009) detected no signals for genetic isolation by distance. Because other factors, for example ecological differences among habitats, also affect the genetic composition of *D. longispina* assemblages (Gießler 1997b; Keller *et al.* 2008), a pattern of clear isolation by distance will be the exception rather than the rule.

We found pronounced year-to-year changes in the composition of *D. longispina* assemblages. In Feldmochinger See, the F1 hybrids were completely replaced by parental *D. galeata* in the following year. Although complete replacements such as this may be rare, fluctuations in the relative taxon frequency have been previously observed in this species complex (e.g. Spaak & Hoekstra 1997; Keller & Spaak 2004). Several experimental studies have shown that taxon fitness varies over time with changes in environmental conditions. For example, Wolf & Weider (1991) reported that F1 hybrids perform better at low temperatures, whereas at high temperatures, parental *Daphnia* are favoured. Another important condition which has a predictable effect on the taxon fitness is the type of predation pressure. Larger taxa (i.e. *D. galeata* vs. hybrids) are selectively removed by visually oriented planktivorous fish (Spaak & Hoekstra 1997), whereas smaller taxa are selected by gap-limited invertebrate predators (e.g. Pijanowska 1990). In contrast to cyclical changes in taxon frequency, which could be easily explained by repeatable, seasonal fluctuations in environmental conditions (e.g. temperature, predation pressure), the taxonomic composition of Feldmochinger See changed dramatically across samples taken at the same time point in subsequent years, just after populations hatched from diapausing eggs. Such rapid replacement, not correlated with seasonal changes has not been reported in the *D. longispina* complex before. A pure taxon effect in the survival of overwintering asexual clones is unlikely as the same parental and hybrid taxa from several other lakes did not respond differently to winter conditions (Rellstab & Spaak 2009). A more likely explanation for the replacement of hybrids by one parental taxon is that the dormant egg bank of parental species is much larger than that of hybrid taxa (Keller & Spaak 2004; Keller *et al.* 2007). Because the population with the larger egg bank is expected to start with more hatchlings at the beginning of the new growing season, they could easily outcompete the hybrids. Alternatively, selection pressures might have changed between years. In two previous studies (Wolinska *et al.* 2006; Brede *et al.* 2009), changes in *D. longispina* taxon composition were also related to long-term directional rather than seasonal change in environmental conditions (i.e. increasing lake trophy, changes in parasite selection pressure). However, the change in taxon composition was relatively slow (lasting a few to dozen years), compared to the rapid change observed here. Further studies are necessary to explore the reasons for this rapid taxon replacement.

Conclusion

Based on 15 microsatellite loci, we studied the genetic diversity of populations from the *Daphnia longispina* complex. We detected replicated multilocus genotypes within populations indicating clonal lineages and also found F1 hybrids having lower genotypic diversity but higher allelic richness. Thus, although hybrid and parental populations both reproduce clonally, we found evidence that the degree of asexual reproduction is much higher in hybrids. In addition, our results show a trend towards isolation by distance for F1 hybrids, suggesting small-scale gene flow in the study region. In one of the studied lakes, there was a rapid year-to-year shift in the taxonomic composition, suggesting significant changes in selection pressure. Finally, we showed that the microsatellites employed in this study can be used for species designation. Altogether, our work shows that the use of microsatellites will provide detailed genetic information on hybrid complexes and will open new windows into understanding the dynamics of hybridizing species, especially in cyclical parthenogens.

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This study was part of the PhD project of Mingbo Yin on the population structure of *Daphnia* species and their common microparasites. Justyna Wolinska is an evolutionary ecologist and her research focuses on the phenomenon of genetic polymorphism and its potential drivers: parasites. Sabine Gießler's major research interest is the dynamics of reticulate evolution and the significance of hybridization in natural systems. All three researchers are using *Daphnia* as a model system.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of 49 reference genotypes, including their sampling location and genetic identification by different marker systems

Table S2 Results of the computation of the most likely number of clusters (K) in STRUCTURE analysis. L(K) and ΔK are likelihoods from 15 simulations. The most likely number of clusters (K = 5) was chosen by highest ΔK

Table S3 Private alleles at 15 microsatellite loci in *D. galeata* and *Daphnia longispina* as defined by CONVERT. Calculations were based on the selection of MLGs from 2008 field samples and reference genotypes. *A*: number of alleles per locus, $A_{T:}$ number of alleles per locus and taxon, $A_{S:}$ number of private alleles per locus, A_r: allelic richness per locus. A_P: the abundance of private alleles per locus. The potentially species-specific alleles (i.e. $A_P > 5\%$ for both taxa) are indicated in bold

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SPATIO-TEMPORAL VARIATION IN *DAPHNIA* COMMUNITY AND POPULATION STRUCTURE– A CASE STUDY OF TWO RESERVOIRS IN CZECH REPUBLIC

Yin M., Petrusek A., Seda J. and J. Wolinska

Submitted

Cyclical parthenogenetic water fleas of the genus *Daphnia* have become a prominent model organism in ecology and evolution. In the past, analyses of their population structure have been limited by the prevailing use of allozyme markers, which in general do not allow for the distinction of individual clones. In this study, we used 10 microsatellite markers to track changes in the taxonomic and clonal composition of *Daphnia* populations in two European reservoirs, and traced the abundance of the most common clones. The study took place during the transition from summer stratification to autumn mixing, representing a period of major environmental change within the lake habitat. One of the studied reservoirs was inhabited by a single species of the *Daphnia longispina* complex (*D. galeata*), the other by two parental species (*D. galeata* and *D. longispina*) and their interspecific hybrids. In both reservoirs, we found temporal (generation-to-generation) and spatial (across the heterogeneous reservoir) changes in *Daphnia* community structure. In the single-species reservoir, the clonal diversity of *D. galeata* increased with time, as a few dominant clones were replaced by a higher number of less common clones. A loss in selective advantage for the dominant clones may

have been due to gradual changes in the environment or to selection acting in a negative frequency-dependent manner. In the multispecies reservoir, there were no apparent temporal trends in clonal diversity but we observed significantly lower clonal diversity in the interspecific hybrids than in the coexisting parental species, supporting the existence of reproductive barriers between the parental genomes. Our study, to trace clonal lineages of *Daphnia* in time and space by the finer marker, contributes to the understanding of how clonal reproduction impacts community structure in cyclically parthenogenetic organisms.

3.1. Introduction

Cyclically parthenogenetic organisms, which reproduce both sexually and asexually, are common in nature, in both the animal and plant kingdoms (Hebert, 1987; Ellstrand et al., 1996). The impact of cyclically parthenogenetic reproduction, especially the asexual (clonal) component, on population structure has attracted considerable interests among population geneticists and evolutionary biologists. For example, in the long-term, clonal reproduction may reduce the number of genetically distinct individuals within a population and hence decrease the effective population size (e.g. Campbell and Husband, 2005). It may also lead to a spatial genetic autocorrelation, which could be falsely attributed to limited propagule dispersal or kin-structured colonization (e.g. Ingvarsson, 1998). Finally, as clones differ in fitness under varying environmental conditions (Vrijenhoek, 1978), changes in clone frequencies are expected across the growing season (Erickson and Hamrick, 2003). Consequently, clonal selection can result in the strong reduction of clonal diversity (Vanoverbeke and De Meester, 2010).

In the freshwater habitats, cyclical parthenogenesis is very common among many groups of

zooplankton (Hebert, 1987). Cladocerans, which reproduce parthenogenetically during favourable conditions and switch to sexual reproduction when conditions deteriorate (e.g. (Hebert, 1978)), are particularly important in these environments, being the main component of aquatic food-webs (Lampert and Sommer, 1999). The cladoceran genus *Daphnia* is commonly used as a model system for cyclical parthenogenesis in ecological and evolutionary research. In some *Daphnia* species complexes, interspecific hybrids may be produced during the sexual part of their reproductive cycle (Hebert, 1985; Schwenk and Spaak, 1995). Hybridization has been documented within several species complexes of *Daphnia* from Eurasia, North America and Australia (Hebert, 1985; Schwenk and Spaak, 1995), but most research has concentrated on the *D. longispina* complex, inhabiting permanent lakes of the northern temperature zone (Schwenk and Spaak, 1995). In Europe, this complex includes, together with some rarer taxa, the widespread and ecologically important species *D. cucullata*, *D. galeata* and *D. longispina* (Petrusek et al., 2008a). These species often form interspecific hybrids which sometimes reach high abundances (Schwenk and Spaak, 1995; 2007; Seda et al., 2007b; Keller et al., 2008; Yin et al., 2010). Once *Daphnia* hybrids are produced by sexual recombination, they can be maintained by clonal propagation for many generations (Schwenk and Spaak, 1995), as in other cyclical parthenogens (e.g. Ellstrand et al., 1996; Delmotte et al., 2003). In the *D. longispina* complex, although parental species also reproduce clonally for most of the year, there is evidence that they invest more into sexual reproduction than F1 hybrids (Keller and Spaak, 2004; Keller et al., 2007).

In previous studies of the *D. longispina* complex, the relative frequencies of different taxa were compared across time (e.g. Spaak, 1996; Jankowski and Straile, 2004; Keller and Spaak, 2004; Brede et al., 2009) and space (e.g. Keller and Spaak, 2004; Seda et al., 2007b; Petrusek et al., 2008c). However, changes in clonal structure have been largely unexplored due to

methodological limitations. So far, the most common method for identification of clones in the *D. longispina* complex has been allozyme electrophoresis (e.g. Ender et al., 1996; Spaak, 1996; Seda et al., 2007b; Rother et al., 2010), although RAPD markers were also used occasionally (e.g. Ender et al., 1996). However, allozyme studies are limited by the few polymorphic loci they provide; in most cases, it is likely that the multilocus genotypes defined by allozymes represented clonal groups (Thielsch et al., 2009). This substantially limits the power to trace the frequencies of single clones and to study clonal structure in general. RAPDs, although more variable, have often poor reproducibility (Devos and Gale, 1992) and, being dominant markers which cannot separate homozygotes from heterozygotes (Suvanto and Latva-Karjanmaa, 2005), have limited use in the analyses of population structure. Recently, microsatellite markers have been developed for the *D. longispina* complex (Brede et al., 2006). However, the subsequent studies employing these markers have focused so far on either a description of population state at a single time point (Thielsch et al., 2009; Dlouhá et al., 2010) or on exploring temporal changes at the taxon level only (Brede et al., 2009; Yin et al., 2010; Rellstab et al., 2011). In other systems, microsatellites have already been proven to be very powerful in tracing clonal lineages; for example, in the cyclically parthenogenetic aphid (Halkett et al., 2005) or in bacterial populations (Imhof and Schlotterer, 2001).

In the present study, we used 10 microsatellite loci to explore temporal and spatial dynamics in the taxonomic and clonal structure of the *D. longispina* hybrid complex, in two reservoirs in the Czech Republic. The canyon-shaped morphology of these reservoirs creates longitudinal environmental gradients, which results in within-locality spatial variation in the composition of zooplankton communities including *Daphnia* (Seda et al., 2007b; Petrusek et al., 2008b). One of the studied reservoirs (Římov) was recently dominated by a single parental species (*D. galeata*), whereas three parental species (*D. cucullata*, *D. galeata*, *D.*

longispina) as well as their interspecific hybrids coexisted in the second reservoir, Vír (Seda et al., 2007b; Petrusek et al., 2008b). We screened *Daphnia* communities in these reservoirs at the end of the growing season, when temperate lakes undergo a major change – a transition from summer stratification to autumn mixing and winter conditions (Lampert and Sommer, 1999). The goals of the study were to explore dynamics in taxonomic and clonal structure, across time (generation-to-generation) and space (between sampling stations along the reservoir's longitudinal gradient), during a period of seasonal environmental change. We also tested one particular hypothesis that the clonal diversity is lower in hybrids than in parental species, due to some pre- and postzygotic barriers between parental genomes (Keller et al., 2007), resulting in a lower number of newly produced hybrids in comparison to parental clones.

3.2. Methods

Study site and field collections

Daphnia samples were collected from two man-made reservoirs in the Czech Republic: Římov (48°50´N, 14°30´E; constructed in 1978) and Vír (49°34´N, 16°19´E; constructed in 1959). Both reservoirs have canyon-shaped morphology, being elongated and meandering in deep valleys (for their outlines, and further morphometric details see Seda et al., 2007b).

We analyzed samples collected from three different stations along the reservoir's longitudinal axis. The first sampling station was always located at the dam and the distance between sampling stations was about 4 km in Římov and 2 km in Vír. The three sampling stations are hereafter referred to as *dam*, *middle* and *upper*. Each station was sampled five times between September 14 (end of the summer stratification period) and December 9, 2009 (onset of winter; see Table 3.S1). The *upper* station in Římov had not been sampled at the last time

point (i.e. *t+4*) because of a very low *Daphnia* density.

Although other factors, such as food availability (e.g. Groeger et al., 1991), can also influences *Daphnia* growth rate, *Daphnia* growth is strongly temperature-dependent (e.g. Spaak and Hoekstra, 1995). Therefore, we adjusted the sampling schedule to one *Daphnia* generation by calculating maturation time based on the surface water temperature (Vijverberg, 1980), using experimental data from Spaak and Hoekstra (1995). Thus, as cooling continued throughout autumn, the sampling intervals became longer. Samples were taken by hauling a plankton net (mesh size 170 µm) through the entire water column, and preserved in 96% ethanol.

Sample processing and microsatellite genotyping

Using the stereomicroscope, ca. 94 adult females from the *D. longispina* complex were randomly chosen from each time point and station for genetic analyses. Additionally, we examined about other 100-350 adult females per sample, for the presence of ephippia, which indicate a switch from parthenogenetic to sexual reproduction. *Daphnia* individuals were incubated in 60-100 µl of H3 buffer with proteinase K for 10-16 hours at 55°C. The H3 buffer included 10 mM Tris-HCl, 0.05 M potassium chloride, 0.005% Tween 20, 0.005% NP-40 and 1.5µg/mL proteinase K (Sigma). Afterwards, samples were heated at 95°C for 12 min to inactive the proteinase K, centrifuged briefly and stored at 4°C.

We applied 10 previously published microsatellite markers (Brede et al., 2006) in a multiplex polymerase chain reaction (Dgm109, Dp196, Dp281, Dp512, SwiD1, SwiD2, SwiD10, SwiD12, SwiD14, SwiD15), by using Multiplex PCR Kit (Qiagen). The forward primers were

labelled with the fluorescent dyes (Applied Biosystems): 6-FAM (Dp196, Dp281, SwiD10), VIC (Dgm109, Dp512, SwiD2, SwiD15), PET (SwiD12, SwiD14), and NED (SwiD1). The amplification protocol consisted of an initial step of 15 min at 95°C to activate the DNA polymerase and denature the template DNA, followed by 33 cycles of 30 sec at 94°C, 1 min 30 sec at 54°C, 1 min at 72°C, and a final extension step of 30 min at 60°C (see Yin et al., 2010). PCR products were analysed on an ABI PRISM 3700 capillary sequencer using a LIZ 500 labelled size standard.

Genotypes were checked by GeneMapper version 3.7 (Applied Biosystems). Alleles at each locus were defined by their fragment length (in base pairs). Before data sets from different plates were merged, the consistency of alleles was checked against loci-specific patterns of a reference clone used in each run, which allowed us to appropriately score alleles with small differences in fragment lengths. In addition, there was no evidence for scoring errors resulted from stuttering, large allele dropout or presence of null alleles, as indicated by tests in MICRO-CHECKER 2.2.3 (10^4 permutations, Van Oosterhout et al., 2004).

Data analyses

Taxon assignment. The similarity of multilocus genotypes (MLGs) characterized by alleles at 10 microsatellite loci was first displayed by the factorial correspondence analysis (FCA), performed in GENETIX 4.05 (Belkhir et al., 1996-2004), in which each different MLG was represented by one individual. As reference parental species, we used 40 well-defined genotypes of *D. cucullata*, *D. galeata* and *D. longispina*, originating from 23 locations in Europe and one location in North America, which were additionally classified using two

allozyme loci, recognized to be diagnostic for species identification (Gießler, 1997a; Dlouhá et al., 2010). For detailed information about those genotypes see (Yin et al., 2010). NewHybrids 1.1 (Anderson and Thompson, 2002), run for 10^6 iterations after a burn-in of 10^6 length, was used to assign individuals from field samples into taxonomic units based on their MLGs. Taxon membership was identified by applying a threshold of 95% posterior probability to assign individuals to one of six predefined categories: two parental species, two backcross groups, or F1 and F2 hybrids. Furthermore, we used logistic regression to test the probability of the distribution of unidentified individuals (below the threshold of 95%) were different among samples; assignment (i.e. indentified vs. unidentified) was treated as a dependent variable and the samples (categorical data) as a covariate.The calculations of clonal diversity within taxa (see below) were based on the taxon assignment from NewHybrids.

Clonal assignment. First, we calculated the P_{sex} index (Stenberg et al., 2003) which determines the likelihood of a clone encountered more than once as being a result of sexual recombination, instead of clonal propagation (GENCLONE 2.0, Haond and Belkhir, 2007). In case of crossing between common clones (or selfing within clone), the likelihood to encounter identical MLGs would be substantially higher than P_{sex} suggests (under random mating); however, this should not change the interpretation of our data, since we assume such genetically similar sibling (if present in our dataset) might also have similar ecological characteristics. Second, in order to assess the resolution power of the ten microsatellite markers used, we tested how many additional multilocus genotypes come out by screening forty individuals from Římov (sampled in autumn 2004) at five additional loci (Dgm105, Dgm112, SwiD5, SwiD7 and SwiD8, as in Yin et al., 2010).

Comparison of clonal diversity between hybrid and parental taxa. Clonal diversity, MLG/N (number of MLG divided by sample size), was calculated for each population sample (defined as a group of individuals belonging to the same taxon, and found at a given time and station) with a minimum sample size of 10 individuals (GENALEX 6, Peakall and Smouse, 2006). All individuals with missing data at any of the loci were excluded (except the *D. longispina* individuals from Vír with missing data at the locus SwiD2; see Results). Then, the clonal diversity was compared between each of the two parental species (*D. galeata* and *D. longispina*) and their F1 hybrids co-occurring at the same time and space (using the Wilcoxon test paired across sampling dates and stations).

Temporal and spatial variation in taxon composition. Using data from Vír, we applied a multinomial generalized linear model (GLM) in R (R Development Core Team, 2009), to test the effects of time (i.e. five time points), space (i.e. three stations) and their interaction term (time × space) on taxon composition; the response was a matrix with five columns (i.e. five classes: *D. galeata*, *D. longispina*, F1 hybrids, backcross to *D. longispina* and unidentified). The command "anova.multinom" was used to perform analyses of deviance. In addition, we tested for the effect of time, space and their interaction on a frequency of a certain taxon (against the frequency of all other taxa in a given sample), by applying the binomial GLM in R. For the model selection, we used a backward elimination procedure, removing the least significant factors ($P > 0.15$) from a parameter-rich model. These analyses were performed for the most common taxa encountered in Vír (*D. galeata*, *D. longispina* and F1 hybrids). Sequential Bonferroni corrections were applied when interpreting the results.

Temporal and spatial variation in clonal composition. First, for both reservoirs, we investigated temporal and spatial changes in clonal composition within taxa. For these analyses, the clones which did not exceed a frequency of 10% in at least one sample of $N \geq 10$, were pooled and labeled as "rare". Similar multinomial GLMs as described above were applied to test the effects of time, space and their interaction (as fixed factors) on clonal composition within taxa and reservoir (i.e. on the response matrix contained all the common clones of the taxon and the "rare" category). Moreover, the binomial GLM was applied to test the frequency of a certain clone against the frequency of all other clones in a given sample (additionally included here were three samples of N = 8 for F1 hybrids and one sample of N = 7 for *D. longispina*). Sequential Bonferroni corrections were applied accordingly.

Temporal and spatial variation in clonal diversity. We used a GLM (family = Gaussian) to test the effects of time, space and their interaction term on clonal diversity (for samples with $N \geq 10$). In order to partition the genetic variance into temporal, spatial and within-sample components, we applied an analysis of molecular variance (AMOVA, $N \geq 10$) in Arlequin 3.0 (10^4 permutations, Excoffier et al., 2005).

3.3. Results

Sexual reproduction

The studied *Daphnia* populations did not switch to sexual reproduction during the study period. Among the 3136 females examined from Vír, only nine (0.3%) females with ephippia were detected. In Římov, not a single sexual female was detected among 2861 examined individuals. Moreover, we only observed a few male animals (even lower proportion than those of phippial females).

Taxon assignment

According to FCA, all 1254 genotyped *Daphnia* from Římov clustered around the reference clones of *D. galeata* (Fig. 3.1a), whereas the 1386 *Daphnia* from Vír clustered around *D. galeata*, *D. longispina*, or in between the two species (Fig. 3.1b). The Bayesian analyses implemented with NewHybrids also assigned MLGs from Římov to one taxon, *D. galeata* (with only one *Daphnia* remaining unidentified with a 95% posterior probability threshold). The MLGs from Vír were assigned to five classes: *D. galeata* (52.6% of all individuals), *D. longispina* (16.5%), backcross to *D. longispina* (3.1%), F1 (20.8%) and F2 hybrids (a single individual; 0.1%). However, 95 individuals (i.e. 6.9%) remained unidentified (Table 3.S2). Even when the threshold of 80% posterior probability was applied, 51 *Daphnia* still remained unidentified; the remaining *Daphnia* were indentified mainly to F1 hybrids (25 individuals) or to *D. longispina* (12 individuals). The unidentified individuals (threshold of 95%) were equally distributed across the samples ($P = 0.16$), and were excluded then from the further analyses.

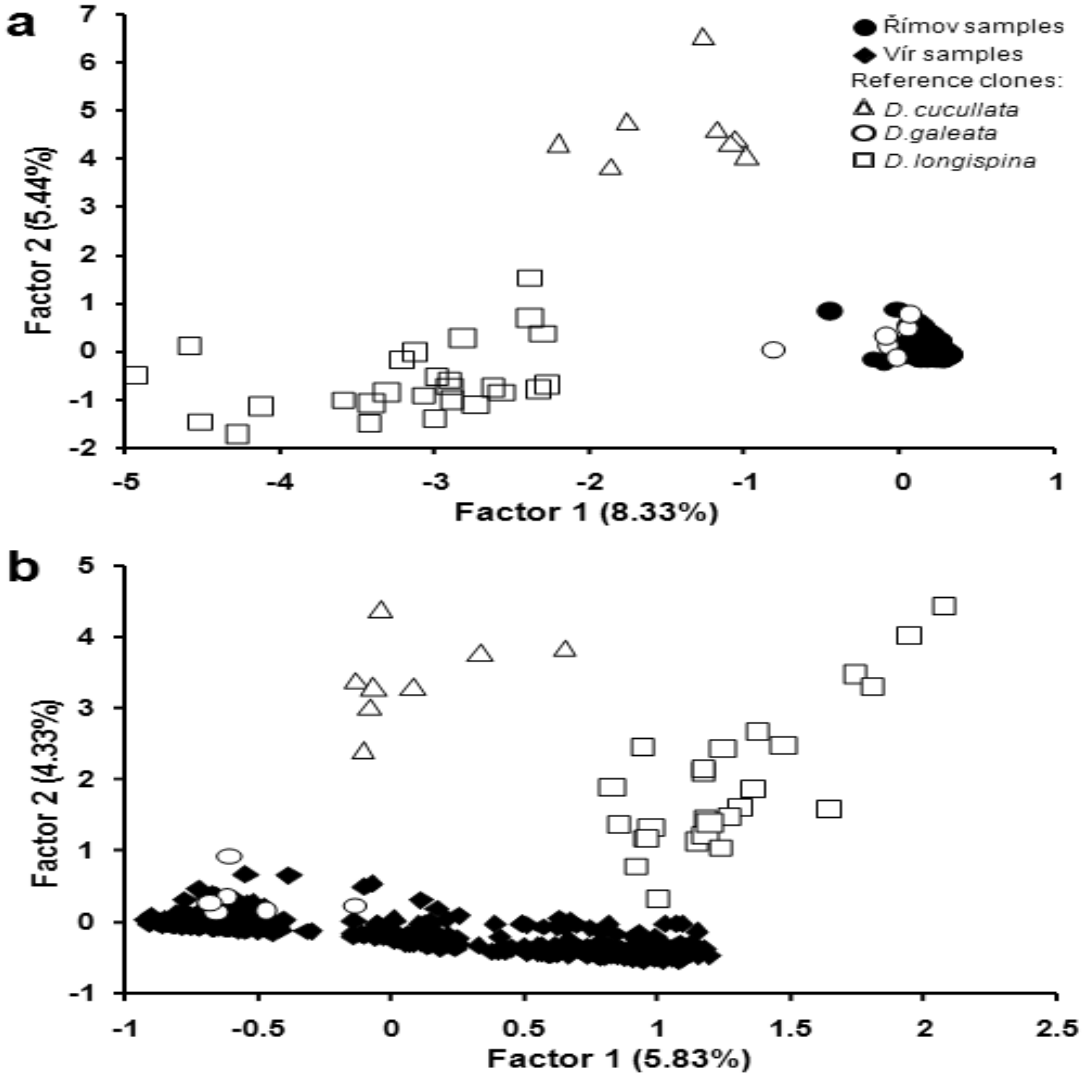

Fig. 3.1. Factorial correspondence analysis showing genetic variation among analysed individuals, based on allelic variation at 10 microsatellite loci. Each data point represents a multilocus genotype from the 40 reference clones and the reservoirs *Daphnia* from a) Římov and b) Vír.

Clonal assignment

In Římov, among the 1220 individuals with complete MLG profiles, 392 unique MLGs were detected. In Vír, among 1329 individuals with complete MLG or data missing solely at the SwiD2 locus (many *D. longispina* individuals could not be amplified at this locus but the amplification worked well at the remaining nine loci), 587 unique MLGs were found. There

was not a single MLG shared between the two reservoirs. As the P_{sex} value was lower than 10^{-5} across all six performed tests (i.e. two reservoirs × three sampling stations), we considered individuals sharing the same multilocus genotype at all evaluated loci as belonging to the same clone. Furthermore, the scoring of the subsamples of the individuals sharing identical MLG at ten loci (8, 5, 4, 3 and 2 individuals, respectively) at five additional loci resulted in the discovery of only one additional MLG (i.e. in case of 2 individuals sharing otherwise the same MLG).

Comparison of clonal diversity between hybrid and parental taxa

In Vír, where two parental taxa and their recombinant genotypes were present, F1 hybrids had significantly lower clonal diversity (MLG/N) than *D. galeata* (0.46±0.15 SD vs 0.74±0.10; N = 12, $Z = 3.06$, $P = 0.002$) or *D. longispina* (0.51±0.13 vs 0.74±0.21; N = 7, $Z = 2.03$, $P = 0.042$; Table 3.S2), which co-occurred with the hybrids at the same time and at the same sampling stations. After excluding the SwiD2 locus, the pattern remained the same (data not shown). Although the sample sizes were smaller for F1 hybrids than for *D. galeata* ($P < 0.001$; paired t-test), sample size did not differ significantly between F1 hybrid and *D. longispina* ($P = 0.46$); thus the variable sample sizes should not be a cause for the observed lower diversity in F1 hybrids.

Temporal and spatial variation in taxon composition

In Vír, the taxon composition of the *Daphnia* community changed significantly across time (i.e. five following *Daphnia* generations) and space (i.e. three stations); whereas the interaction effect was only marginally significant (multinomial GLM, Table 3.S3). Looking at changes in the frequencies of single taxa (vs. other taxa, binomial GLMs), the frequencies of

D. galeata and *D. longispina* changed across time and space, whereas the frequencies of the F1 hybrids changed across time only and there was a significant time × space interaction (Fig. 3.2, Table 3.1). Interestingly, the observed temporal changes in *D. galeata* were opposite between *dam* and *middle* stations (see marginally significant time × space interaction: Table 3.1). In the *dam* station, the proportion of *D. galeata* decreased from 60% to 17%, between *t+1* and *t+2* (Fig. 3.2a). In contrast, the proportion of *D. galeata* increased from 24% to 60% during the same time period at the *middle* station (Fig. 3.2b).

Table 3.1. Changes in relative taxon frequency across time and space in Vír. The fitted GLM model is shown (terms removed from the model are labelled as 'ns'). The *P*-values that remained significant after sequential Bonferroni correction are marked in bold (α = 0.05/3).

	Time		Space		Time × Space	
Taxon	*Z*	*P*	*Z*	*P*	*Z*	*P*
D. galeata	-2.42	**0.015**	3.12	**0.002**	1.73	0.084
F1 hybrids	2.18	0.029		ns	-3.80	**<0.001**
D. longispina	3.71	**<0.001**	-7.76	**<0.001**		ns

Temporal and spatial variation in clonal composition

In order to trace the clonal lineages, seven *D. galeata* clones from Římov, and four *D. galeata*, five F1 hybrid and two *D. longipina* clones from Vír were selected as a set of "common" clones (with frequency exceeding 10% in at least one sample), whereas the other clones were pooled together into the "rare" group (Fig. 3.3). *D. longispina* from Vír were only analysed at the *dam* and *middle* stations because of their low frequency at the *upper* station (see Fig. 3.2). For *D. galeta* from Římov and the three taxa from Vír, significant changes in clonal composition were detected across time (i.e. five subsequent *Daphnia* generations; $P < 0.01$), space (i.e. three stations; $P < 0.01$) and their interaction (i.e. time × space; $P < 0.05$; Table 3.S4).

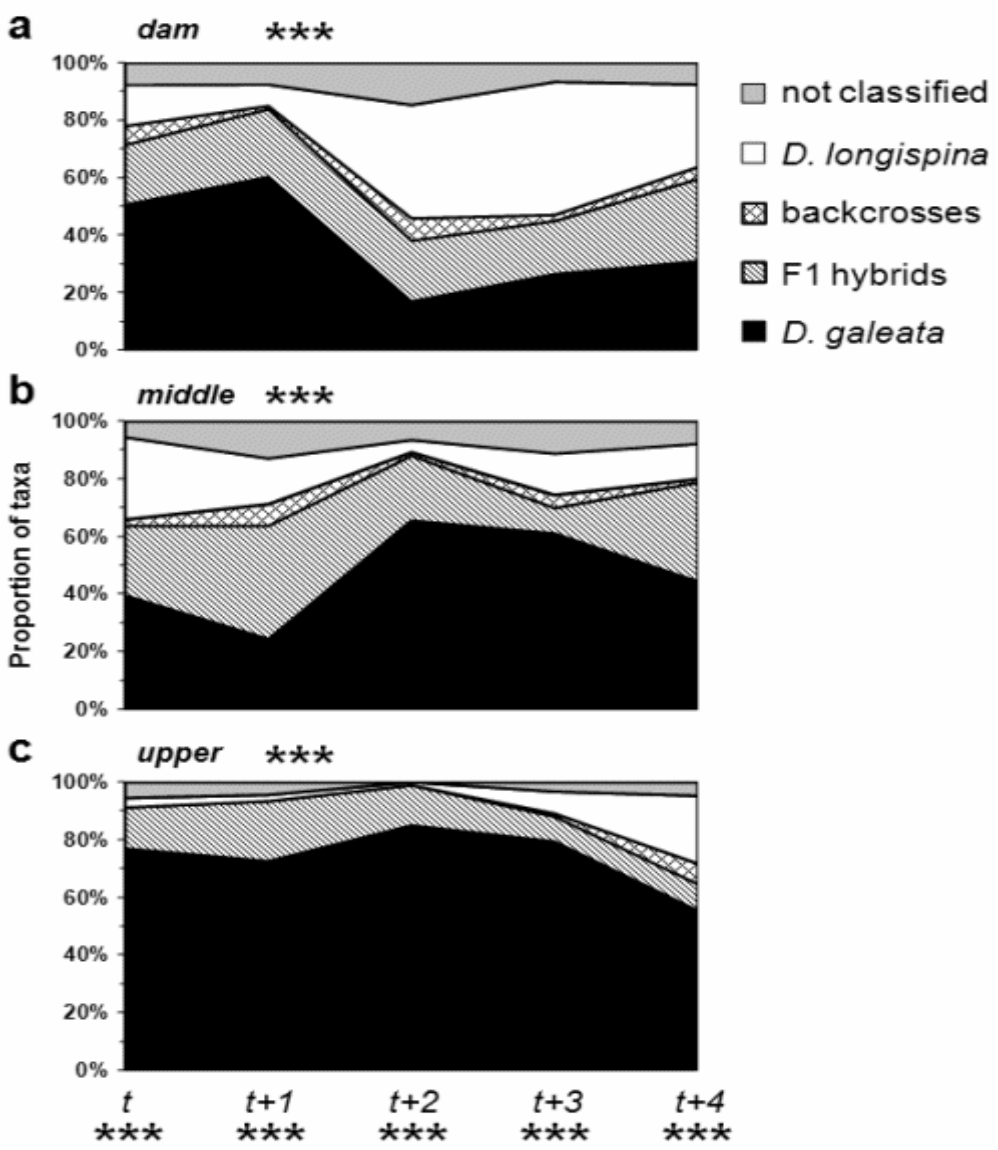

Fig. 3.2. Changes in the taxon composition of *Daphnia* communities in the Vír reservoir at each of the three sampling stations: a) *dam*, b) *middle* and c) *upper*. Taxon classification is based on the NewHybrids assignment; a single F2 hybrid is pooled with unidentified individuals (below a threshold of 95% posterior probability).

In the binomial GLM model, six *D. galeta* from Římov, as well as three *D. galeata*, three F1 hybrid and two *D. longipina* clones from Vír were analysed (the remaining "common" clones were only detected at one or two sampling dates / stations, resulting in too many zero values to be included in the dataset). In Římov, four out of the six tested common *D. galeata* clones showed significant changes across time, five clones across space, and three clones showed a

significant time × space interaction (Table 3.2). For example, the proportion of clone 4 at the *dam* station remained constant at ca. 25% for the first two time points (i.e. at *t* and *t+1*) and then decreased to below the detection limit at *t+3* (i.e. 42 days later; Fig. 3.3a). In the *middle* station, the proportion of clone 4 increased from 14% at *t* to 46% at *t+1* (i.e. within 15 days), then decreased and fluctuated afterward (around 15%; Fig. 3.3b). In the *upper* station, the proportion of clone 4 was much lower and fluctuated with time (from below detection to 10%; Fig. 3.3c). In the *D. galeata* population from Vír, one of the three common clones showed significant changes across time and space and a significant interaction effect (Table 3.2). Among the F1 hybrids, one of the three tested clones showed significant changes across time and space; specifically, clone I dominated the population at time *t* in both the *middle* (59%, Fig 3.3h) and *upper* stations (69%, Fig. 3.3i), but was rare at the *dam* station (10%, Fig. 3.3g). The frequency of this clone consistently decreased towards the end of the growing season at all three stations. A second clone differed across time only, whereas a significant time × space interaction was detected for a third clone (Table 3.2). Among the *D. longispina* clones, one of the two tested clones showed significant changes across time, and both clones showed a significant interaction effect (Table 3.2).

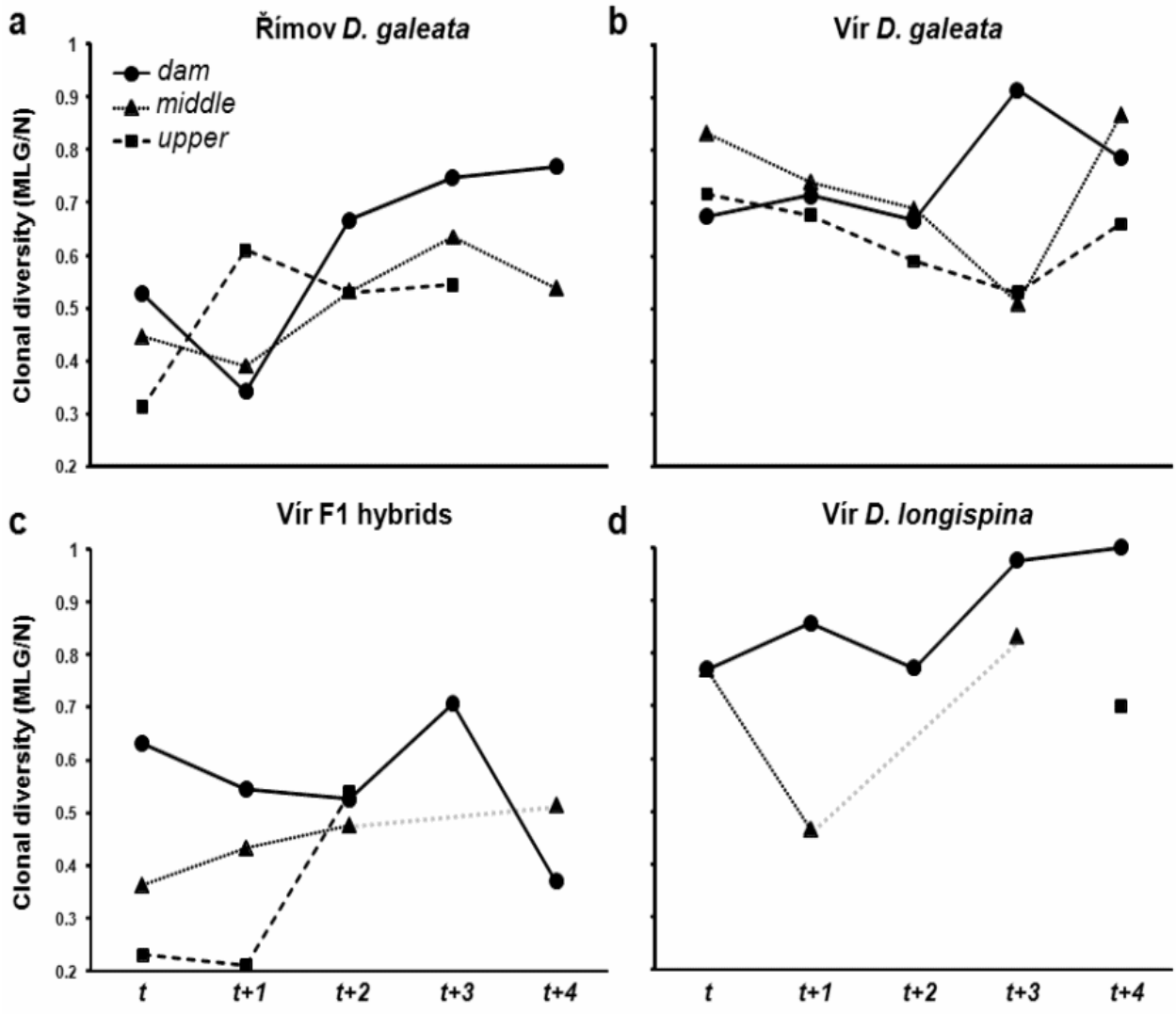

Fig. 3.3. Changes in clonal composition of *Daphnia* taxa in Římov and Vír reservoirs at each of the three sampling stations (*dam*, *middle* and *upper*). Distribution of the most common clones (i.e. frequency > 10% in at least one sample) is shown. Remaining clones were pooled and classified as "rare" (white area, up to 100%). NA indicates dates when *Daphnia* were no longer present at the sampling site. A blank square across the graph indicates a date when there were too few individuals of the species available to calculate clone frequencies (n = 4).

Temporal and spatial variation in clonal diversity

In the Římov reservoir, the clonal diversity of the *D. galeata* population increased significantly with time (Fig. 3.4a, Table 3.3). In the Vír reservoir, however, there was no apparent trend in any of the three tested taxa (Fig. 3.4b-d). Even after pooling all the taxa into an "all-*Daphnia*" dataset, no temporal trend in clonal diversity was detected (data not shown). In the F1 hybrids, the clonal diversity differed significantly among stations; it was low at the *upper* station, intermediate at the *middle* station and high at the *dam* station (Fig. 3.4c).

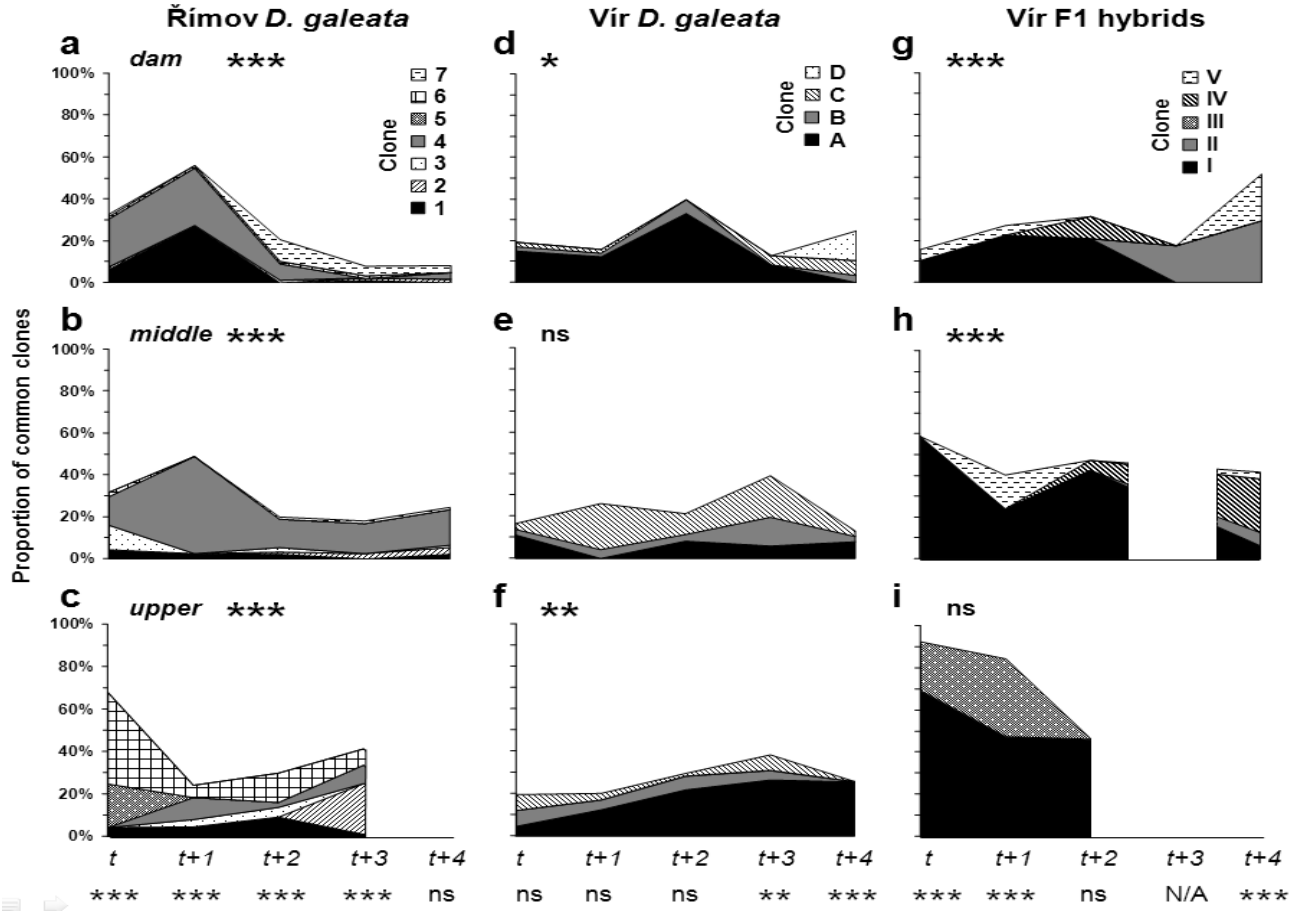

Fig. 3.4. Changes in clonal diversity (number of distinct multilocus genotypes / sample size) of a) *D. galeata* in Římov and b) *D. galeata*, c) F1 hybrid and d) *D. longispina* populations in the Vír reservoir, at each of the three sampling stations (*dam*, *middle* and *upper*). Only samples with n ≥ 10 are shown, grey dotted lines connect results from non-adjacent sampling dates.

The AMOVA indicated that most of the genetic variance was explained by within-sample variation (from 91% to 99%, Table 3.4). The remaining variation was more or less equally distributed between temporal and spatial components. These components were in all but one case significant; the exception being a spatial component for *D. galeata* in Vír. This corresponded well to the results of the binomial GLM tests (see above), in which clonal frequency differed in space for one of the three common *D. galeata* clones only (Table 3.2).

Table 3.2. Changes in relative frequencies of common clones across time and space, in Římov and Vír (calculated per *Daphnia* taxon). The fitted GLM model is shown (terms removed from the model are labelled as 'ns'). The *P*-values that remain significant after sequential Bonferroni correction are marked in bold (α = 0.05/6; /3; /3; /2; respectively).

		Time		Space		Time × Space	
Taxon (reservoir)	clone ID	*Z*	*P*	*Z*	*P*	*Z*	*P*
D. galeata (Římov)	1	-3.38	**<0.001**	-2.87	**0.004**	2.10	0.035
	2	-2.83	**0.004**	-2.66	**0.007**	3.73	**<0.001**
	3	-2.83	**0.005**		ns	1.88	0.059
	4	-5.52	**<0.001**	-5.48	**<0.001**	4.73	**<0.001**
	6		ns	8.52	**<0.001**	-5.57	**<0.001**
	7		ns	-3.46	**<0.001**	1.99	0.05
D. galeata (Vír)	A	-3.39	**<0.001**	-2.93	**0.003**	4.10	**<0.001**
	B		ns	1.28	0.198		ns
	C	0.51	0.613		ns		ns
F1 hybrids (Vír)	I	-5.21	**<0.001**	4.04	**<0.001**		ns
	IV		ns	-2.07	0.04	3.07	**0.002**
	V	2.51	**0.012**		ns	-2.13	0.03
D. longispina (Vír)	a	-2.72	**0.006**	-1.83	0.07	2.32	**0.020**
	b	-1.93	0.052		ns	3.12	**0.001**

3.4. Discussion

Using a set of recently developed high-resolution microsatellite markers (Brede et al., 2006), we observed changes in *Daphnia* taxon and clonal composition and diversity on a very fine temporal and spatial scale, for two *Daphnia* communities. Specifically, the sampling intervals were adjusted to a single *Daphnia* generation (i.e. 14-30 days, depending on temperature) and the spatial distribution of taxa and clones were studied within individual water bodies.

Table 3.3. Changes in clonal diversity across time and space, in Římov and Vír (calculated per *Daphnia* taxon). All significant values are marked in bold.

	Time		Space		Time × Space	
Taxon (reservoir)	*F*	*P*	*F*	*P*	*F*	*P*
D. galeata (Římov)	7.78	**0.018**	1.76	0.21	0.07	0.79
D. galeata (Vír)	0.03	0.86	2.90	0.11	1.93	0.19
F1 hybrids (Vír)	0.67	0.43	13.49	**0.006**	9.05	**0.016**
D. longispina (Vír)	2.08	0.22	1.91	0.24	0.01	0.94

In contrast to the Vír reservoir, where two parental species (*D. galeata* and *D. longispina*) and three hybrid classes (backcrosses, F1 and F2) were detected, only one taxon (*D. galeata*) was found in Římov. These results correspond to previous allozyme-based studies of *Daphnia* communities in the same reservoirs (Seda et al., 2007b; Petrusek et al., 2008c). Differences in the *Daphnia* taxon composition between the environmentally similar habitats are not extraordinary: both the coexistence of several taxa from the *D. longispina* complex and the dominance of one taxon have been reported across many European lakes (e.g. Spaak, 1996; Keller et al., 2008; Yin et al., 2010). In Vír, we observed temporal and spatial taxonomic shifts during the seasonal transition. The observed shifts may be explained by the impact of

alternations in environmental conditions on the relative fitness of taxa. Previous experimental studies on the *D. longispina* complex have shown that relative taxon fitness varies with food quality (e.g. Seidendorf et al., 2007), predation (e.g. Spaak et al., 2000) and parasite pressure (Wolinska et al., 2006). Indeed, differences in local food conditions and predation regimes are considered as drivers of spatial differentiation in taxon composition within reservoirs (Seda et al., 2007b; Petrusek et al., 2008c). Moreover, we have recently observed that parasite pressure also varies across time and space in these reservoirs (Wolinska et al., 2011).

Table. 3.4. Hierarchical analysis of molecular variance (AMOVA) among sampling stations and within time points (calculated per *Daphnia* taxon).

Taxon(reservoir)	Source of variation	DF	Percentage of explained variation	*P*
D. galeata (Římov)	Across space	2	0.95	**< 0.05**
	Across time (within space)	11	4.13	**< 0.001**
	Within sample	2494	94.9	**< 0.001**
D. galeata (Vír)	Across space	2	0.00	0.297
	Across time (within space)	12	0.93	**< 0.001**
	Within sample	1425	99.1	**< 0.001**
F1 hybrids (Vír)	Across space	2	2.64	**< 0.001**
	Across time (within space)	9	1.45	**< 0.010**
	Within sample	508	95.9	**< 0.001**
D. longispina (Vír)	Across space	2	5.53	**< 0.001**
	Across time (within space)	6	2.92	**< 0.001**
	Within sample	395	91.6	**< 0.001**

In addition to affecting the distribution of *Daphnia* species and hybrids, environmental heterogeneity may also affect competition at the clonal level. Although some clones were shared among the sampling dates and stations, their relative frequencies differed (similar as reported in a recent study by using allozyme markers, Frisch and Weider, 2010). This is in

agreement with the experimental studies on the *D. longispina* complex which revealed that the relative performance of clones varies across environmental conditions, even within a single taxon (e.g. Spaak et al., 2000; Wolinska et al., 2006; Seidendorf et al., 2007). Moreover, for one taxon (F1 hybrids in Vír), the clonal diversity also differed across the stations in a consistent manner; being high at the *dam*, intermediate at the *middle* and low at the *upper* station. Various scenarios may explain such patterns. First, some clones detected at the *dam* station could be passively dispersed from the upper parts of the reservoir; i.e., the dam region might serve as a sink that accumulates more genotypes. Second, there is an environmental gradient within the reservoir even at the end of the season and spatial differences in local condition could promote different clonal diversity in some taxa.

In general, populations of cyclical parthenogens are expected to have high clonal diversity at the start of the growing season due to the hatching of new genotypes. Conversely, during asexual reproduction, the extinction of clones due to selection and random events should lead to an erosion of clonal diversity (De Meester et al., 2006; Vanoverbeke and De Meester, 2010). For example, allozyme studies have detected a decrease in clonal diversity in *D. magna* and *D. pulex* populations inhabiting temporary ponds during the course of a single growing season (Ruvinsky et al., 1986; Carvalho and Crisp, 1987) and similar patterns were reported for other cyclical parthenogens such as rotifers (e.g. Ortells et al., 2006) and aphids (Sunnucks et al., 1997). However, for the transition period between summer stratification and autumn mixing, which happens at the end of the growing season, our data showed an increase in clonal diversity with time for the *D. galeata* from Římov, and clonal diversity remained roughly constant in *D. galeata*, *D. longispina* and hybrids from Vír. This suggests that changes in selection pressures during this period did not result in further clonal erosion.

The observed decrease in the frequency of some common clones (which might result in an increase in clonal diversity) could potentially result from an investment into sexual rather than parthenogenetic reproduction. However, this was not the case in the studied reservoirs; as the proportion of ephippial females and males was negligible. Rather, it seems that in Římov, a few *D. galeata* clones that were dominant at the beginning of autumn lost their relative competitive advantage and were later replaced by a higher number of otherwise less common clones. The clones that were favoured during stratified summer conditions may have relatively lower fitness in low food and low temperature, while other clones may be better adapted to these harsh environments. For example, a recent study found the presence of a genetically differentiated hypolimnetic population of *D. galeata* in Římov (Seda et al., 2007a) with different life-history traits than their epilimnetic counterparts (Machacek and Seda, 2008). Such clones originating from the deep hypolimnion, where the water is colder and less nutrient-rich, may have an advantage at the end of the growing season. The decrease in the abundance of common clones could also be caused by selection pressure acting in a negative frequency-dependent manner. For example, there is some evidence from a field survey of the *D. longispina* complex communities that common clones are attacked by coevolving parasites, which consequently reduce their frequencies (Wolinska and Spaak, 2009). As the prevalence of *Daphnia* parasites is high in both reservoirs, especially in autumn (Wolinska et al., 2011), it is possible that common clones are handicapped by parasite-driven, time-lagged, negative frequency-dependent selection (see Jokela et al., 2009).

Finally, the significant differences in clonal diversity between co-occurring taxa in Vír supports our hypothesis that F1 hybrids have lower clonal diversity than parental species. This is consistent with the results from a recent field survey of *D. longispina* communities across several small lakes in Germany, where the clonal diversity of F1 hybrids was lower than that

of parental species (Yin et al., 2010). In that previous study, however, clonal diversity was compared between the taxa originated from different water bodies, so it could not be excluded that the observed patterns were partially caused by habitat differences. In another field survey of a *D. longispina* community across a single lake, significantly fewer hybrid genotypes were detected in sexually-produced diapausing eggs (epphipia) than would be expected if mating were random; furthermore, hybrid embryos were shown to have lower hatching success than parental embryos (Keller et al., 2007). Thus, the present study contributes strong evidence for the existence of reproductive incompatibilities between the parental genomes of hybridizing species in the *D. longispina* complex.

3.5. Conclusions

By applying a fine-scale design we have shown significant temporal and spatial changes in taxonomic and clonal composition in communities of the *D. longispina* hybrid complex. Analysis of 10 microsatellite loci allowed us to trace clonal lineages with unprecedented precision, in contrast to previous studies using very broad, allozyme-defined clonal groups (e.g. Jacobs, 1990; Spaak, 1996; Gießler, 1997b; Anderson and Thompson, 2002; Rother et al., 2010). On the one hand, our data show the replacement of dominant clones over a very short time period (within one or two generation times) and spatial genetic differentiation within single water bodies. On the other hand, we detected the presence of certain clones in substantial frequencies at sampling stations separated by several kilometres. Apparently, successful genotypes reach high densities and occupy vast areas within the reservoir despite the variation in selection pressure. Most likely, these common clones overwinter in the reservoir, which allows them to compete with other genotypes for extended periods of time. Altogether, our work highlights detailed changes in clonal structure within the *D. longispina*

hybrid complex and contributes to understanding how clonal reproduction impacts community composition in cyclically parthenogenetic organisms.

3.6. Acknowledrments

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Chapter 4

DAPHNIA POPULATIONS INFECTED WITH TWO VIRULENT PARASITES – HOST GENETIC STRUCTURE ON A SMALL TEMPORAL AND SPATIAL SCALE

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Submitted

Numerous theoretical studies suggest that parasites impose a strong selection pressure on their host, driving genetic changes within host populations. Yet evidence of this process in the wild is scarce. In the present study we surveyed, using high resolution microsatellite markers, the genetic structure of cyclically parthenogenetic *Daphnia* hosts within two different *Daphnia* communities belonging to the *Daphnia longispina* hybrid complex. One community, consisting of a single host species, was infected with the protozoan parasite *Caullerya mesnili*. The second community consisted of two parental *Daphnia* species and their hybrids, and was infected with the yeast parasite *Metschnikowia* sp.. Significant differences in the clonal composition between random and infected sub-samples of *Daphnia* were detected on several occasions within both communities, indicating that host genotypes differ in resistance to both parasites. In addition, one parental species in the multi-taxon community was consistently under-infected, compared to the other taxa. Overall, our field data confirm that infections have genetic background in the *Daphnia*-microparasite systems. Thus, parasite-driven selection operates in natural *Daphnia* populations, and microparasites influence the clonal

structure of host populations.

4.1. Introduction

Theoretical work suggests that parasites impose a strong selection pressure on their hosts, driving genetic changes within host populations (e.g. Jaenike, 1978; Hamilton, 1980). A core mechanism in determining the effect of parasites on their host populations is the non-random infection of host genotypes, such as when parasites target the common host genotypes in a population (Red Queen hypothesis, Bell, 1982) which in turn lose their fitness advantage and drop in frequency (i.e. negative frequency-dependent selection, Hamilton, 1980; Lively, 1996). As a result of this process, the genotypic composition of the infected proportion of a population should be significantly different from the composition of the population as a whole (e.g. Little and Ebert, 1999; Jokela et al., 2009). Further, if infected and uninfected individuals differ in their reproductive success, this can result in selection and eventually in shifts of genotype frequencies. Such parasite-mediated selection has been detected under laboratory conditions (Buckling and Rainey, 2002; Haag and Ebert, 2004; Lohse et al., 2006; Koskella and Lively, 2009), as well as in some natural host populations (Woolhouse et al., 2002; Decaestecker et al., 2007; Duncan and Little, 2007; Jokela et al., 2009; Wolinska and Spaak, 2009).

Natural populations of parthenogenetic or cyclical parthenogenetic hosts are excellent models to study parasite-driven selection. Such populations consist of clonal lineages and, by applying variable molecular markers to field collected samples, it is possible to link the frequencies of certain genotypes with their infection level, and to follow changes in the frequency of individual genotypes over time. This method has been successfully applied to populations of freshwater snails (Dybdahl and Lively, 1998; Jokela et al., 2009) and to the

crustacean waterflea *Daphnia* (Little and Ebert, 1999; Wolinska and Spaak, 2009). *Daphnia* can be infected by a variety of microparasites, including fungi, bacteria, protozoa and microsporidia (Green, 1974; Ebert, 2005). Moreover, many parasite species are lethal to or sterilize their *Daphnia* hosts (e.g. Hall et al., 2005; Wolinska et al., 2007a). This high level of virulence may result in parasites driving genetic polymorphism within host population (Howard and Lively, 1994). Changes in host population structure can be relatively quick (Duncan and Little, 2007; Wolinska and Spaak, 2009) as *Daphnia* generation time is on average only 1-2 weeks. Finally, the prevalence of parasites varies across (Ebert et al., 2001; Hall et al., 2005; Wolinska et al., 2011) and even within natural *Daphnia* populations (Wolinska et al., 2011), providing the opportunity to study host population structure under various degrees of parasite selection pressure.

So far the most common method for identifying clones in *Daphnia*-parasite studies has been allozyme electrophoresis (Little and Ebert, 1999; Wolinska et al., 2004; Duncan and Little, 2007; Wolinska and Spaak, 2009). However, only a limited number of polymorphic loci can be analysed by this method due to limited tissue availability. Moreover, the number of alleles per locus tends to be relatively low in allozyme markers (Leberg, 1992). It is thus likely that a multilocus genotype as defined by allozymes represents a clonal group rather than an individual clone (Thielsch et al., 2009). This questions the reliability of the method in tracing changes in frequencies of single genotypes. In the present study, we applied microsatellite markers to compare the genetic structure between random and infected parts of the host population from the *Daphnia longispina* hybrid complex. Microsatellite markers, having much higher resolution than previously used allozymes, have been successfully applied to tracking clonal lineages in *Daphnia* (e.g. Pantel et al., 2011), but not yet employed in the context of parasite-driven selection.

We compared the genetic structure between random and infected sub-samples of *Daphnia* populations originated from two water reservoirs in the Czech Republic. In one of these reservoirs, *Daphnia* were infected with the protozoan parasite *Caullerya mesnili* (class Ichthyosporea, Lohr et al., 2010b). In the other reservoir, the yeast *Metschnikowia* sp. (family Hemiascomycetes, Wolinska et al., 2009) was the dominant microparasite. *Caullerya* lives in the gut epithelium of *Daphnia*, whereas the spores of *Metschnikowia* fill the entire *Daphnia* body cavity. Across European lakes, both parasites commonly infect populations of various taxa of the *D. longispina* complex, including the hybrids (Wolinska et al., 2007a; 2009; 2011). Both parasites were found to be highly virulent to their *Daphnia* host; for example, *Caullerya* reduces host fecundity by 95% (Wolinska et al., 2007a) whereas *Metschnikowia* - by 25% to 50% (Duffy et al., 2008; Hall et al., 2009). Moreover, both parasites decrease host life span (Lohr et al., 2010a). Regarding a genetic specificity of infection, which is an important prerequisite for the Red Queen (Seger and Antonovics, 1988; Agrawal and Lively, 2002), laboratory studies have shown *C. mesnili* has high degree of host specialisation. This parasite infects various host taxa and genotypes from the *D. longispina* hybrid complex to different extent (Wolinska et al., 2006; Schoebel et al., 2011). At the same time, there is lack of consistency regarding the evidence of genetic specificity in the *Daphnia-Metschnikowia* system. Although some experimental studies reported differential infection rate among the tested host genotypes (Duffy et al., 2011), others did not (Yin et al., 2011). In the latter one, different taxa and clones within the *D. longispina* complex were infected to similar extent in a laboratory experiment.

We made the following predictions in our study: i) the genetic composition should differ between random and infected sub-samples of *Daphnia* populations when infected with *Caullerya*, due to host specialisation. It is unclear if such patterns should be also observed in

populations infected with *Metschnikowia*, as there are contrasting evidence regarding genetic specificity of host-parasite interactions in that system. ii) Consequently, in the *Caullerya*-infected populations (and, maybe, in the *Metschnikowia*-infected populations), the evenness (and clonal diversity) of infected sub-samples should be lower than the evenness of random sub-samples.

4.2. Materials and methods

Study site and field sampling

We collected *Daphnia* samples from two drinking-water reservoirs in the Czech Republic, Římov and Vír. The reservoirs are ~154 km apart and they are located in different river basins. However, they share basic characteristics – a single main river tributary, elongated canyon-shaped morphology, stratified water column along most of the reservoirs length, and maximal depth over 40 m (for detailed description of reservoirs see Seda et al., 2007b). Both reservoirs are inhabited by *Daphnia* from the *D. longispina* complex: Římov is dominated by a single parental species (*D. galeata*), whereas in Vír three parental species (*D. cucullata*, *D. galeata*, *D. longispina*) as well as their interspecific hybrids have been detected (Seda et al., 2007b; Petrusek et al., 2008c). For each reservoir we analysed *Daphnia* samples collected from three different stations along the reservoirs longitudinal axes. The first sampling station was always located at the dam, and the distance between sampling stations was about 4 km in Římov and 2 km in Vír. The three sampling stations are hereafter referred to as dam, middle and upper.

We sampled each station five times between September 14th and December 9th, 2009 (see Table 4.S1). This is the period when the reservoirs undergo the transition from summer stratification to winter conditions and is characterised by an increased prevalence of both studied microparasites (Wolinska et al., 2011). Also, during autumn *Daphnia* reproduce only

clonally in the studied reservoirs, as only nine females carrying sexual eggs and even less male individuals were detected among 5997 screened *Daphnia* (Yin et al., unpublished data). *Daphnia* growth is strongly temperature-dependent (e.g. Spaak and Hoekstra, 1995), although other factors, such as food availability (Groeger et al., 1991), can also influence *Daphnia* growth rate. Thus, sampling intervals were adjusted to one *Daphnia* generation by calculating maturation time (Vijverberg, 1980) based on water temperature and experimental data from Spaak and Hoekstra (1995). Samples were taken by hauling a plankton net (mesh size 170 μm) through the entire water column. The samples were preserved in 96% ethanol.

Sample selection

In total, 29 samples were analysed (the densities of *Daphnia* in the upper station of Rimov reservoir were very low at time *t+4*; this sample has not been considered then). To choose the most prevalent parasite in the reservoirs, we screened ca. 100-300 adult female *Daphnia* from each time point and sampling station. Because of *Daphnia*'s transparent body, infections are easily detectable with a stereomicroscope (magnification up to ×250), without dissecting the host. For any sample where a parasite was highly abundant we randomly selected 94 adult females (hereafter referred to as 'random sample') and an additional sub-sample of 47 infected females ('infected sample'), for further genetic analyses.

Genotyping

We genotyped all individuals at ten microsatellite markers (Dgm109, Dp196, Dp281, Dp512, SwiD1, SwiD2, SwiD10, SwiD12, SwiD14, SwiD15, Brede et al., 2006) in a fixed multiplex PCR (according to the method described by Yin et al., 2010). PCR products were analysed on an ABI PRISM 3700 capillary sequencer using a LIZ 500 labelled size standard. Allele sizes were checked in GeneMapper version 3.7 (Applied Biosystems, Germany). Alleles at each

locus were defined based on the bp length of fragments. Before data sets from different plates were merged, we checked the consistency of alleles with loci-specific patterns using a reference clone in each run. The multilocus genotype (MLG) structures assessed by these ten microsatellite markers are a good approximation of the true clonal structure in the studied *Daphnia* populations. Specifically, the scoring of *Daphnia* individuals from Římov sample sharing identical MLG at ten loci (i.e. eight, five, four, three and two individuals, respectively) resulted in the discovery of only one additional MLG at five additional loci (i.e. in case of two individuals sharing otherwise the same MLG, Yin et al., unpublished data).

Data analyses

Differences in taxon composition between random and infected samples.

Taxon membership was identified using NewHybrids 1.1 (Anderson and Thompson, 2002) by assigning each individual, based on the highest posterior probability, to one of six predefined categories: two parental species, two hybrid (F1 and F2) and two backcross classes (10^6 iterations after a burn-in of length 10^6). Since only a few individuals were assigned to the backcross or F2 classes, these were pooled with the F1 hybrids into a more general category called 'hybrids'. We used logistic regression to determine the probability that the taxon with the most pronounced difference in frequency between random and infected samples was indeed under or over-infected compared to other taxa; taxon (i.e. one vs. others) was treated as a dependent variable and the sample type (i.e. random and infected) as a covariate.

Differences in clonal composition, clonal diversity and evenness between random and infected samples.

Only individuals with complete MLG profiles, or occasionally, *D. longispina* individuals with missing data at a single locus, SwiD2, were included. The calculations were performed when

a minimum of 20 individuals per taxon were available from a given time point and station. First, the clonal composition was compared between random and infected samples, per given time point and station. We used a Monte Carlo approach with 10^5 simulation runs (Sham and Curtis, 1995). For these analyses, clones that did not reach a threshold frequency of 5.0% in random or infected samples were pooled into the 'rare' category. As two to three time points were tested per station, we applied sequential Bonferroni corrections (Rice, 1989) while interpreting the results. Second, we compared clonal diversity (calculated as MLG/N in GENALEX 6, Peakall and Smouse, 2006) and clonal distribution (i.e. the evenness index E_{var}, Smith and Wilson, 1996) between random and infected samples using Wilcoxon matched pair tests.

4.3. Results

Sample selection based on parasite prevalence

The most prevalent parasites were *C. mesnili* in Římov and *Metschnikowia* sp. in Vír. No *Metschnikowia* was detected in the Římov samples. *Caullerya* was present in Vír but at two sampling dates only, and with a low prevalence (< 3%; the prevalence data is shown in Table 4.S2). As the parasites did not have high enough prevalence at all sampling dates (Table 4.S2), it was only possible to collect and analyse the following infected samples: Římov *Daphnia* infected with *Caullerya* at three time points (*t+1*, *t+2* and *t+3*) and at three different stations (dam, middle and upper) - resulting in nine paired comparisons (three time points × three stations); and Vír *Daphnia* infected with *Metschnikowia* at two time points (*t+2* and *t+3*) and three different stations (dam, middle and upper) – resulting in six comparisons, respectively.

Taxon composition of random and infected sample

All 1179 individuals from Římov (pooled from both random and infected samples) were classified into one taxon (*D. galeata*), whereas the 825 individuals from Vír were assigned to one of three classes: *D. galeata*, *D. longispina* or hybrids; predominantly F1 but with occasional presence of F2 and backcross genotypes (third parental taxon, *D. cucullata*, is only present in early summer, Petrusek et al., 2008c). For Vír, in which multiple host taxa were present, *D. galeata* were under-infected by *Metschnikowia* in comparison with other taxa (Wald = 22.5, d.f = 1, $P < 0.001$; Fig. 4.1). When similar test was performed for hybrids - they were over-infected in comparison with other taxa (Wald = 13.2, d.f = 1, $P < 0.001$; Fig. 4.1). No over- or under-infection was found for *D. longispina* (Wald = 4.1, d.f = 1, $P = 0.05$; Fig. 4.1).

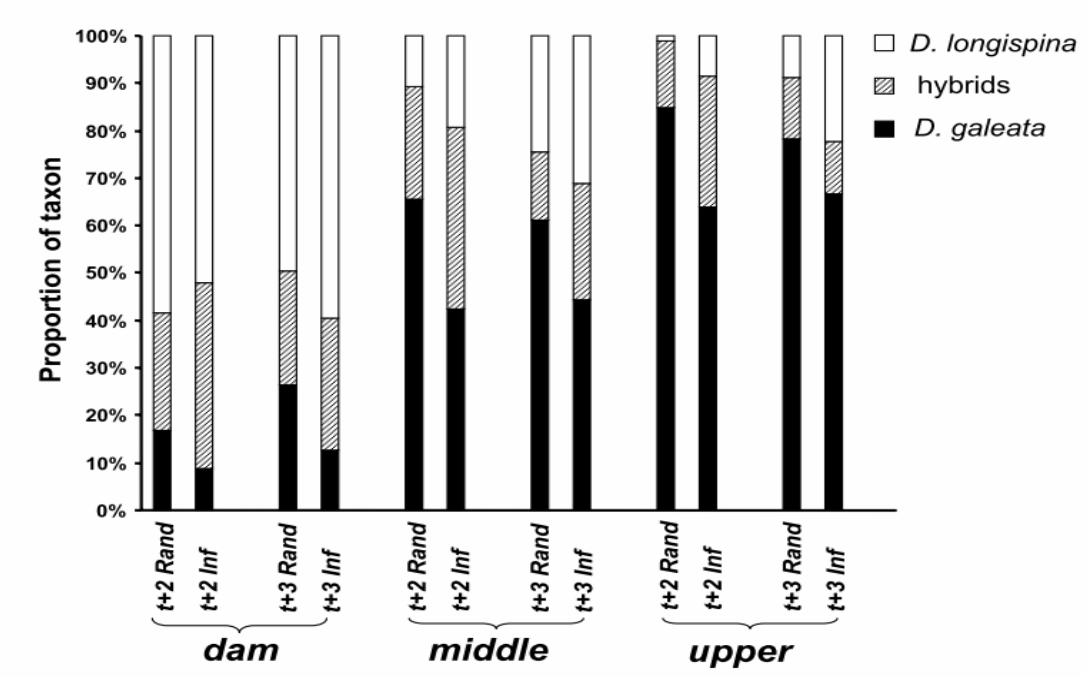

Fig. 4.1. Comparison of taxon composition between random (Rand) and *Metschnikowia*-infected (Inf) samples in Vír, at three sampling stations (dam, middle and upper) and two different time points (*t+2* and *t+3*).

Clonal composition of random and infected samples

In Římov, the clonal composition differed significantly between random and *Caullerya*-infected *D. galeata* in three out of nine comparisons, as assessed by Monte Carlo simulations

(Fig. 4.2A). In Vír, the clonal composition was significantly different between random and *Metschnikowia*-infected samples in one out of four comparisons for *D. galeata* (Fig. 4.2B) and in both comparisons for *D. longispina* (Fig. 4.2C). The sample size for the hybrid group was too low to perform further statistical tests.

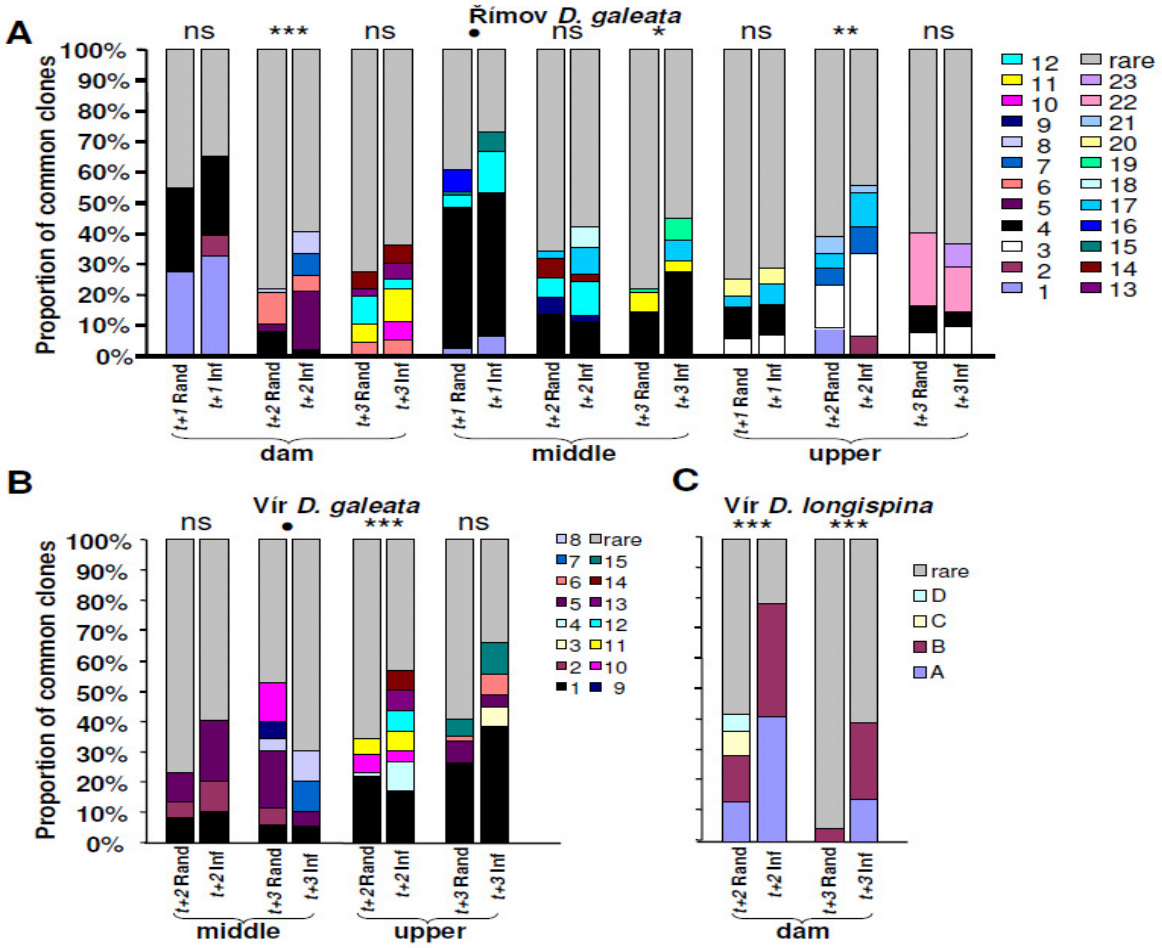

Fig. 4.2. Comparison of clonal composition between (A) random (Rand) and *Caullerya*-infected (Inf) samples of *D. galeata* in Římov, (B) random and *Metschnikowia*-infected samples of *D. galeata* in Vír and (C) random and *Metschnikowia*-infected samples of *D. longispina* in Vír; at one to three sampling stations (dam, middle, upper) and two or three different time points (*t+1*, *t+2*, *t+3*). The 'rare' category (grey) was composed of clones that did not reach the 5 % threshold frequency in the random or infected samples. ** P <0.01; *** P <0.001 (after sequential Bonferroni correction); • significant difference was only detected before sequential Bonferroni correction; ns - not significant.

Clonal diversity and evenness of random and infected samples

In Římov, the clonal diversity did not differ between random and *Caullerya*-infected *D. galeata* samples (Wilcoxon matched pair tests: N = 9, Z = -1.72, *P* = 0.086; Fig. 4.3A; Table 4.S2). The same was true in Vír where the clonal diversity did not differ between random and *Metschnikowia*-infected samples, as calculated per taxon (*D. galeata*: N = 4, Z = -1.46, *P* = 0.14; *D. longispina*: N = 2, Z = -1.34, *P* = 0.18, Fig. 4.3B, C; Table 4.S2). Similarly, there was no difference in the distribution of clones (evenness index, E_{var}) between random and *Caullerya*-infected samples (*D. galeata*: N = 9, Z = -0.77, *P* = 0.44; Fig. 4.3A) or between random and *Metschnikowia*-infected samples (*D. galeata*: N = 4, Z = -1.46, *P* = 0.14; *D. longispina*: N = 2, Z = -1.34, *P* = 0.18, Fig. 4.3B, C).

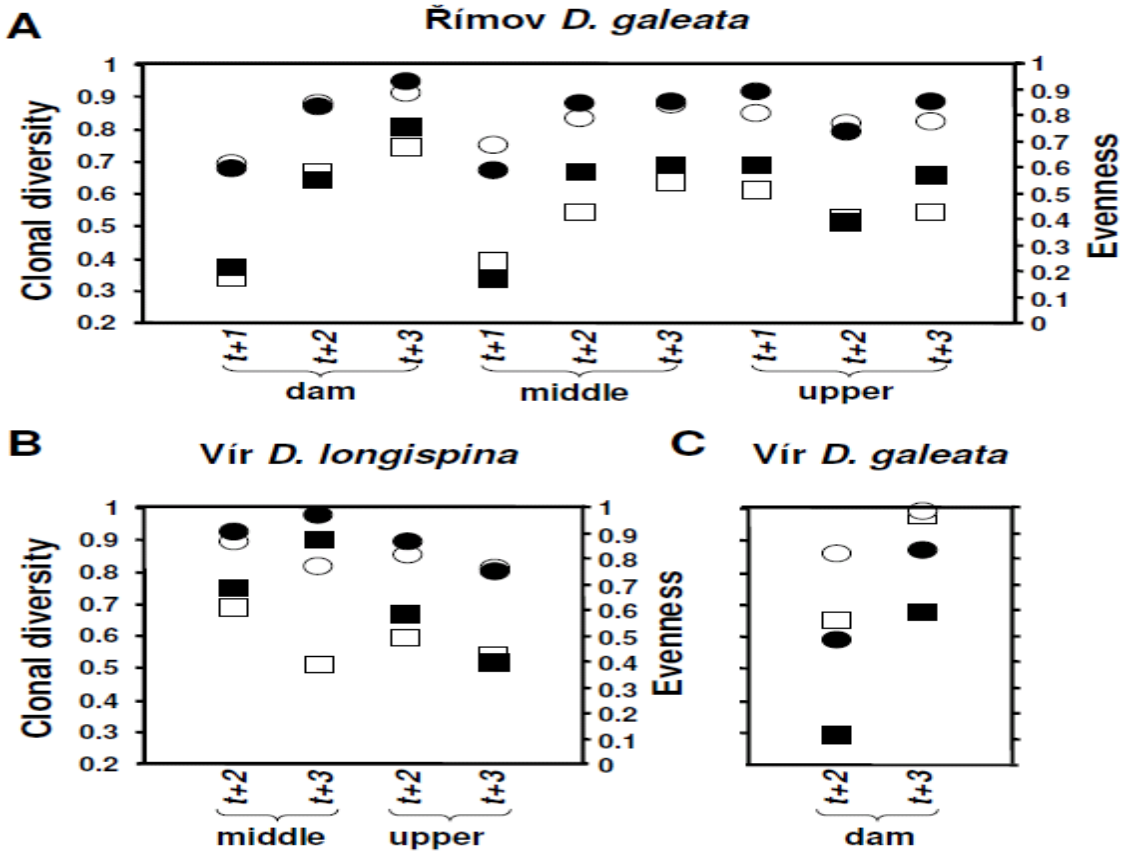

Fig. 4.3. Comparison of clonal diversity (MLG/N; squares) and evenness (E_{var}; circles) between (A) random (empty symbols) and *Caullerya*-infected (filled symbols) samples of *D. galeata* in Římov, (B) random and *Metschnikowia*-infected samples of *D. galeata* in Vír and (C) random and *Metschnikowia*-infected samples of *D. longispina* in Vír; at one-three sampling stations (dam, middle and upper) and two-three different time points (*t+1*, *t+2* and *t+3*).

4.4. Discussion

Parasite-driven, negative frequency-dependent selection can operate if parasites evolve to infect common host genotypes (e.g. Bell, 1982; Nee, 1989). Such adaptations and the respective selection against infected genotypes may result in different genotypic compositions between random and infected parts of the host population. In particular, if parasites are virulent enough (Lively, 1999) they may cause a decrease in the frequency of the most common genotypes over time (Duncan and Little, 2007; Jokela et al., 2009; Wolinska and Spaak, 2009). Based on a set of high-resolution microsatellite markers, we compared the genetic structure between random and infected sub-samples of *Daphnia* populations in order to explore if parasites are an important selection factor in populations of this key planktonic grazer. Consistent with our expectations, we detected significant differences in clonal composition between random and *Caullerya*-infected parts of the host population at several occasions. Similar differences were detected in *Daphnia* populations infected with another parasite, *Metschnikowia*, which was sometime reported to be a generalist (e.g. Stirnadel and Ebert, 1997; Yin et al., 2011). These results provide additional evidence that parasite-driven selection might operate in natural *Daphnia* populations (e.g. Little and Ebert, 1999; Duncan and Little, 2007; Wolinska and Spaak, 2009).

Previous field studies of *Daphnia longispina* hybrid communities infected with *Caullerya* showed that the relative infection of host taxa differed across time (Wolinska et al., 2006) and space (Wolinska et al., 2007a), highlighting the dynamic nature of host-parasite interactions. For example, in the extensively studied *Daphnia* community of lake Greifensee in Switzerland, *Daphnia galeata* was first under-infected by *Caullerya*, in relation to hybrids, but over-infected two years later, after a strong increase in frequency (Wolinska et al., 2006). Thus, an observed change in relative taxa infection was consistent with the idea of parasite

evolution towards the most frequent host taxon (Wolinska et al., 2006). Consequently, it is not unlikely that the observed here, in Vir, under-infection of *D. galeata* (or over-infection of hybrids) by *Metschnikowia* is only temporary, as was the case for the *Daphnia-Caullerya* system (Wolinska et al., 2006).

The significant differences in genotype composition (assessed by allozyme markers) between the random and infected parts of host population have been detected in several previous studies; in *Daphnia* (Little and Ebert, 1999; Little and Ebert, 2000; Wolinska et al., 2004) or freshwater snails (Koskella and Lively, 2009). In the present work, we also detected significant differences in clonal composition between random and infected part of host (*Daphnia*) populations; in six out of fifteen performed comparisons (three out of nine cases with *Caullerya,* and three out of six with *Metschnikowia*). This indicates that both parasites infect different host genotypes to various extent. In the above-mentioned surveys (i.e. on *Daphnia* and snails) the assignment of individuals to certain clones was based on allozyme profiles. It can be argued thus that the 'clone' was in reality a grouping of genotypes sharing the same allozyme-profile (Thielsch et al., 2009). The results of this study, using microsatellite markers with substantially higher resolution, show that the previously observed associations were indeed real, and suggest that parasite-driven selection is common in natural *Daphnia* populations.

For neither parasite, however, was clonal diversity or evenness different between the random and infected sub-samples. If parasite populations rapidly adapt to the common host genotype (Red Queen hypothesis, Bell, 1982), it is expected that the evenness (and clonal diversity) of infected sub-samples should be lower than the evenness of random sub-samples. However, the presence of too many rare clones in our samples may have obscured this pattern.

Compared with previous allozyme-based studies of host-parasite interactions in *Daphnia* (Little and Ebert, 1999; Wolinska et al., 2004; Duncan and Little, 2007; Wolinska and Spaak, 2009), we were able to achieve better resolution for testing the association between the genetic composition of random and infected samples of host populations. In the future, use of these highly variable markers should be the standard approach in the detection and tracking of individual genotypes for host-parasite studies. This will provide better insights into the real dynamics of infections within host genotypes.

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PREDATOR-INDUCED DEFENCE MAKES *DAPHNIA* MORE VULNERABLE TO PARASITES

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PREDATOR-INDUCED DEFENSE MAKES *DAPHNIA* MORE VULNERABLE TO PARASITES

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Inducible defensive traits against herbivores or predators are widespread in plants and animals. Theory predicts that defended morphs have greater fitness in the presence of predators, but lower fitness than undefended morphs in the absence of predators. If such costs did not exist, then a constitutively defended morph would be favored by natural selection; yet, evidence for such costs has been elusive. Our current work reveals a significant cost to inducible defenses. Using the waterflea (*Daphnia*) model system, we show that induced defended morphs are significantly more vulnerable to infection by a virulent yeast parasite than undefended morphs. In two independent experiments, the proportion of successful infections and the number of parasite spores were higher among defended versus undefended *Daphnia*. Thus, by demonstrating a previously unknown and environmentally relevant cost to inducible defenses, this study enhances our understanding of adaptive phenotypic plasticity and its evolution.

KEY WORDS: **Inducible defenses, kairomones, *Metschnikowia*, phenotypic plasticity.**

Predation is a major driver of natural selection. Phenotypic plasticity for the induction of defensive traits has proven to be a widespread mechanism for plants and animals to cope with the risk of consumption by herbivores and predators (Agrawal 2001). On theoretical grounds, inducible defenses are most likely to evolve when there is spatial or temporal heterogeneity in predation, and when there are reliable cues that can be used to indicate the future risk of attack (Lively 1986; Riessen 1992; Frank 1993; Hazel et al. 2004). Present models for the evolutionary stability of induced defense suggest that there must be a cost associated with the defended morph in the absence of predators; otherwise we would expect the defense to be constitutively expressed (Lively 1986; Van Tienderen 1991; Clark and Harvell 1992; Moran 1992; Riessen 1992; Frank 1993; Hazel et al. 2004). Nonetheless, empirical studies in some systems have revealed only weak costs (e.g., Tollrian 1995; DeWitt 1998; Scheiner and Berrigan 1998; Van Buskirk 2000; Relyea 2002; Van Kleunen and Fischer 2005; Van Buskirk and Steiner 2009; Auld et al. 2010). For example, life-table experiments conducted on the rotifer *Brachionus calyciflorus* showed that neither the possession of long spines nor the production of offspring with long spines interfered with rotifer survivorship, fecundity, or reproductive potential (Gilbert 1980).

It has been suggested that the primary cost of inducible defenses is a reduction in growth and development (Clark and Harvell 1992; Tollrian and Harvell 1999). For example, tadpoles build large tails to escape predators, but this comes at the cost of slower growth (meta-analysis in Van Buskirk 2000). Small planktonic waterfleas (*Daphnia*) with induced neck spines (called neck-teeth) have an approximately 5–15% delay in reproduction compared to undefended morphs (Black and Dodson 1990; Riessen and Sprules 1990). However, these observations of growth and developmental costs have been questioned. Tollrian (1995) argued that the increased time to maturity is not the result of a direct physiological cost but a trade-off for larger body size. Consequently,

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by studying the way morphological and life-history traits respond to fish cues in a set of *Daphnia* clones, Boersma et al. (1998) concluded that morphological defenses are often independent of developmental shifts. Thus, the commonly observed reductions in growth and developmental rates may not represent costs in the formation of morphological defenses, but trade-offs between adaptive changes in life-history parameters.

Alternatively, it has been suggested that the evolution of inducible defenses may be favored by alterations in the abundance of predators with different hunting strategies, which may create mismatches between induced defenses and predation risk (Tollrian and Harvell 1999). For example, freshwater snails can either produce invasion-resistant shells for defense against water bugs or crush-resistant shells for defense against crayfish, but not both (Hoverman et al. 2005). Likewise, waterfleas are trapped in the same paradoxical situation. In the presence of fish, which feed selectively on larger prey (Brooks and Dodson 1965), *Daphnia* reproduce at an earlier age (at smaller size) and produce a greater number of small offspring (Stibor and Lüning 1994; Boersma et al. 1998). When gape-limited invertebrate predators are the most dominant threat, however, *Daphnia* react in the opposite way; by delaying reproduction and producing larger offspring (Stibor and Lüning 1994; Riessen 1999). Consequently, if the relative abundance of different enemies changes, then the induced phenotype becomes a handicap rather than an advantage.

Despite numerous studies investigating these costs in light of adaptation to the wrong form of predation (reviewed in Tollrian and Harvell 1999), the potential costs of prey exposure to enemies from different functional levels, predators and parasites, remain insufficiently explored. This lack of knowledge is surprising as nearly all organisms are affected, to some extent, by parasitism. For example, recent work shows that food webs, previously regarded to be built on predator–prey relationships, are incomplete without the incorporation of parasites (Lafferty et al. 2008). It has even been argued that parasitism is the most common animal life style (Lafferty et al. 2006).

Daphnia provide a textbook example of inducible life-history defenses against predators (reviewed in Tollrian and Dodson 1999; Laforsch and Tollrian 2009) and, at the same time, they are known to face strong pressure from multiple parasites in nature. Given that parasites are often highly virulent to their *Daphnia* host (reviewed in Ebert 2005), a higher susceptibility of defended *Daphnia* to infection would represent an environmentally relevant cost. Among the hundreds of predator–prey and host–parasite experimental surveys using *Daphnia* as the target prey or host, only three studies have investigated the consequences of simultaneous exposure to both stressors: predator cues (kairomones) and parasite spores (Lass and Bittner 2002; Coors and De Meester 2008, 2010). In none of these studies, however, was the prevalence of infection compared between the predator-defended and undefended morphs. This is because the authors focused either primarily on the host, but not on the parasite fitness (Lass and Bittner 2002; Coors and De Meester 2008), or because each individual *Daphnia* became infected, independently of the kairomone-exposure treatment (Coors and De Meester 2011), probably due to extremely high infectious doses. Thus, the potential trade-offs between these two selection pressures, which would be of high ecological and evolutionary relevance, remain unknown.

In the study described here, we raised individuals from multiple clones, representing two *Daphnia* taxa, in the presence or absence of fish kairomones, and subsequently exposed them to parasite spores. We predicted that the kairomone treatment would induce life-history changes known to improve fitness in the presence of predators (Tollrian and Dodson 1999), but that the predator-induced individuals would show greater infection prevalence and/or intensity than the individuals not exposed to kairomones.

Materials and Methods

STUDY SYSTEM

Host (*Daphnia longispina* complex, as revised in Petrusek et al. 2008) and parasite (*Metschnikowia* sp., Fig. 1) were isolated from Ammersee, Germany, in 2008. Two *D. galeata* and three *D. galeata* × *D. longispina* hybrid clones were selected from a larger collection of sampled clones, to represent two *Daphnia* taxa inhabiting lake Ammersee (Yin et al. 2010) and different multilocus genotypes within each taxon (as assessed at 15 microsatellite loci, for methods see Yin et al. 2010). The parasite strain was isolated by exposing one of the clones (Clone_53) to a spore extract from crushed, lake-infected *Daphnia*. Stock

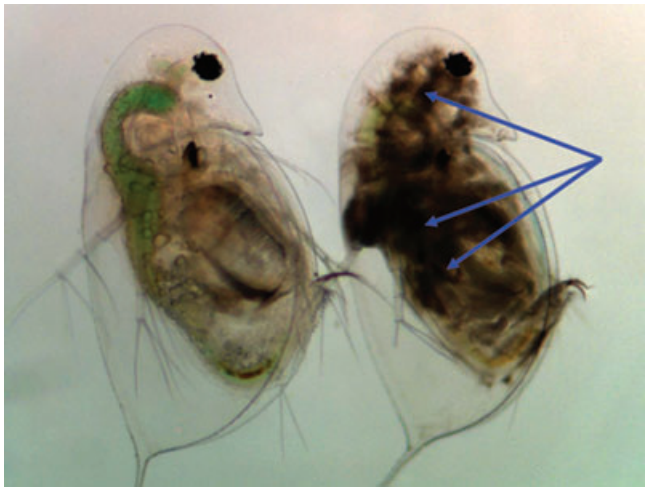

Figure 1. Image of noninfected (left-hand side) and heavily infected *Daphnia* hosts (from *D. longispina* complex). These animals were kept in the control media. Arrows indicate regions of high spore density.

parasite cultures were maintained by adding uninfected *Daphnia* every second week (Lohr et al. 2010). Both infected and uninfected *Daphnia* cultures were kept in synthetic media (based on ultrapure water, trace elements and phosphate buffer), at 20°C, 16:8 light-dark photoperiod, and fed three times per week with 1.0 mg CL^{-1} unicellular algae, *Scenedesmus obliquus*.

MEDIA PREPARATION

Two types of media were used: (1) fish kairomone, and (2) control medium. The former was obtained by keeping one fish (bitterling *Rhodeus sericeus amarus*) in a 5-L aquarium for 22 h. A fish was fed ~25 *Daphnia* per day (mixture of all clones used in the experiments). The term kairomones is used to refer to all chemicals directly (i.e., infochemicals) or indirectly (i.e., alarm cues released by *Daphnia*, Laforsch et al. 2006) associated with the presence of fish. All media were filtered before use (0.2 μm filter), using a separate filter to avoid contamination.

EXPERIMENTAL DESIGN

A full factorial design of two independently run experiments included: medium (kairomones/control), parasite exposure (presence/absence), and host identity (different clones). In Experiment 1, five clones were tested with 10 replicates per clone (except Clone_66 with 6 replicates only). In Experiment 2, a reduced number of clones were used (three instead of five) but the number of replicates in the parasite-presence treatments was doubled.

EXPERIMENTAL PROCEDURES

Prior to the experiment, nine adult *Daphnia* females (per clone) were randomly selected from mass cultures. From these mothers, 24 neonates were collected and passed through two subsequent generations to remove maternal effects, before serving as the mothers of the experimental animals. During this period, *Daphnia* were kept individually in 80 mL of medium and fed daily with 1.0 mg CL^{-1} of *S. obliquus*. The temperature and light conditions were the same as for the stock cultures (see above).

The experimental neonates (third brood; born within a 48 h span) were randomly assigned to different treatments. On day 1, they were placed individually into 5 mL of kairomone or control media. The medium was refreshed daily (except days 6 and 8). At day 5, half of the experimental units were exposed to parasite spores: laboratory-infected *Daphnia* were crushed (12 and 15 individuals in Experiments 1 and 2, respectively) and a spore extract was added at a concentration of 700 spores/mL (Lohr et al. 2010). To avoid possible bacteria or alarm cue effects, crushed uninfected *Daphnia* were added as a placebo treatment in Experiment 2 (at similar concentration as infected *Daphnia*; see above). The infection procedure was repeated on day 7. *Daphnia* were fed daily with 1.0 mg CL^{-1}, except day 4 and 6 (the starvation period prior to infection was shown to increase the uptake of spores, Hall et al. 2007). On day 9, *Daphnia* were transferred to 30 mL of fresh kairomone or control medium, respectively. Offspring were removed and counted daily. Dead *Daphnia* were ground up and the number of mature spores (Lohr et al. 2010) was determined using a Neubauer improved counting chamber. The experiment lasted 24 days.

DATA ANALYSIS

Prevalence data were analyzed using logistic regression, whereas all other traits were analyzed using analysis of variance (ANOVA, SPSS 15.0). All independent variables (i.e., medium, parasite exposure, and host identity) were treated as fixed factors. In the analyses of host traits (i.e., age at first reproduction and number of offspring in the first clutch) only infected animals were included in the parasite-presence treatments. Because of the lower number of replicates in Experiment 1, host traits were analyzed in Experiment 2 only. Data were tested for normality and homogeneity of variance using a Kolmogorov–Smirnov and Levene's test, respectively. Variables that did not conform to normality were transformed using the Rankit function (Conover and Iman 1981)

Results

Kairomone exposure induced life-history defenses

In the presence of kairomones, *Daphnia* reproduced earlier and produced more offspring in the first clutch (Fig. 2). Moreover, infected *Daphnia* matured earlier than noninfected *Daphnia*, but only in the control treatment (medium-by-parasite interaction: $P = 0.026$, Table 1). Infected *Daphnia* produced less offspring, regardless of the kairomone treatment. All clones reacted in a similar way (Table 1).

Defended morphs were more vulnerable to infection

In two independently run experiments, the prevalence of infection (Fig. 3A) as well as the parasite spore load (Fig. 3B) were higher in *Daphnia* exposed to kairomones. This was true for all clones tested (Table 1).

Discussion

We found that induced *Daphnia* suffer a cost of defense by becoming more susceptible to parasite infection. We are certain that we compared the susceptibility to parasites between defended and undefended host phenotypes, as exposure to chemical cues released by fish caused typical life-history responses. Similar to many previous studies (e.g., Stibor and Lüning 1994; Boersma et al. 1998), fish-induced *Daphnia* produced more offspring and reproduced at an earlier age. Thus, in two independently run experiments, both the proportion of successful infections and the number of

Table 1. The effects of medium (kairomone/control), parasite exposure (presence/absence) and host identity (different clones) on various host and parasite fitness measures. Prevalence data were analyzed using a logistic regression, whereas all other traits were analyzed using ANOVA.

Dependent variable	Experiment	Source of variation	df	F/Wald χ^2	P
Age of reproduction	No. 2	Medium	1	5.30	0.026
		Parasite	1	0.05	0.827
		Clone	2	0.90	0.414
		Medium × parasite	1	5.30	0.026
		Clone × parasite	2	2.00	0.148
		Clone × medium	2	1.93	0.158
		Clone × medium × parasite	1	0.07	0.796
		error	41		
Number of offspring (1st clutch)	No. 2	Medium	1	6.17	0.016
		Parasite	1	7.67	0.008
		Clone	2	2.93	0.061
		Medium × parasite	1	0.66	0.421
		Clone × parasite	2	0.60	0.551
		Clone × medium	2	1.83	0.170
		Clone × medium × parasite	2	2.61	0.082
		error	58		
Prevalence of infection	No. 1	Medium	1	5.36	0.021
		Clone	4	2.19	0.700
		Clone × medium	4	3.72	0.054
	No. 2	Medium	1	10.62	0.001
		Clone	2	2.00	0.370
		Clone × medium	2	2.17	0.337
Intensity of infection	No. 1	Medium	1	20.54	<0.001
		Clone	4	0.39	0.816
		Clone × medium	4	2.08	0.104
		error	35		
	No. 2	Medium	1	11.98	0.001
		Clone	2	0.33	0.723
		Clone × medium	2	0.49	0.619
		error	49		

parasite spores were, on average, twice as high among defended morphs; strikingly, the pattern was consistent across all *Daphnia* clones tested. Coors and De Meester (2011) also compared the number of parasite spores per infected host, between kairomone-exposed and control *D. magna*. Contrary to our results the presence of fish kairomones decreased the average amount of bacterial (*Pasteria ramosa*) spores. However, only a single *Daphnia* clone was tested in their experiment (Coors and De Meester 2011).

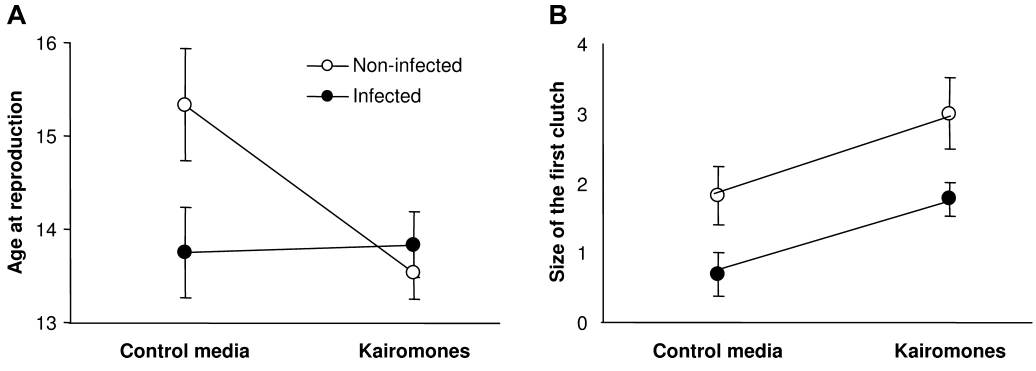

Figure 2. Comparison of *Daphnia* fitness components; *Daphnia* were either exposed to kairomones released by fish or kept in control media. In addition, *Daphnia* were either infected (with a yeast parasite *Metschnikowia* sp.) or healthy. (A) Age of reproduction (mean ± SE). (B) Number of offspring in the first clutch (mean ± SE).

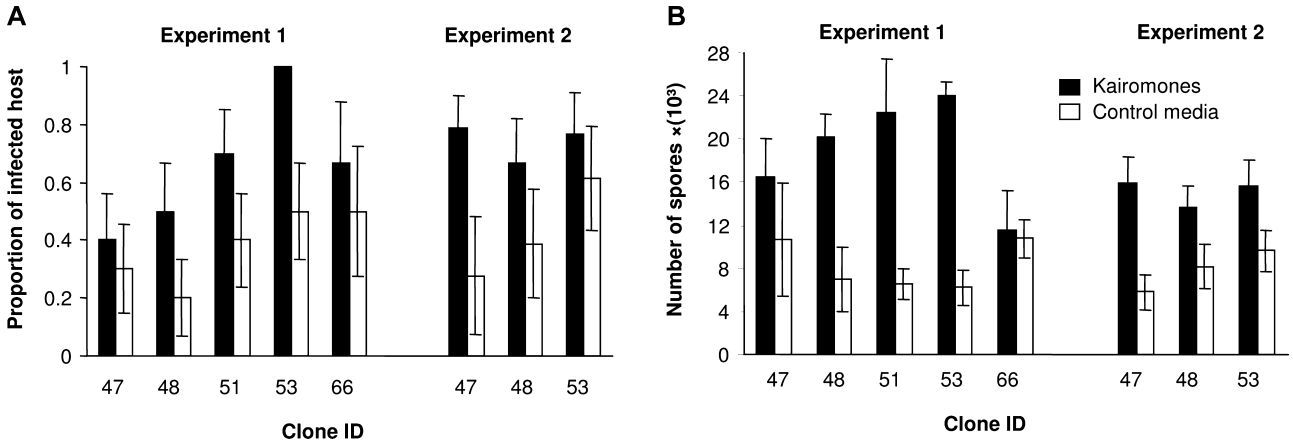

Figure 3. **Comparison of *Daphnia* susceptibility to the yeast parasite, *Metschnikowia* sp.: *Daphnia* were either exposed to kairomones released by fish or kept in control media. The experiment was repeated with a reduced number of *Daphnia* clones (five in Experiment 1 and three in Experiment 2). (A) Proportion of *Daphnia* infected (mean ± SE). (B) Number of mature parasite spores per infected *Daphnia* at death (mean ± SE).**

Given the strong genetic variability of *D. magna* in response to fish predation (Boersma et al. 1998) as well as to *P. ramosa* infection (Decaestecker et al. 2007), the findings of Coors and De Meester (2011) might be specific to the *Daphnia* genotype tested in their experiment.

The *Daphnia* taxa used in our study (belonging to the *D. longispina* complex) are common inhabitants of permanent European lakes (e.g., Keller et al. 2008; Yin et al. 2010). Similarly, the studied parasite, *Metschnikowia* sp., frequently infects natural *D. longispina* populations. For example, the parasite was detected in four out of 11 *D. longispina* populations screened in the Czech Republic (Wolinska et al. 2009, 2011). The parasite prevalence in those populations reached up to 38%, indicating that the experimental parasite doses used in the present study are not unlikely in nature. Importantly, these two selective pressures, fish predators and the *Metschnikowia* parasite, are to some extent, separated in time. *Metschnikowia* sp. peaks in autumn, and is undetectable in other seasons (Duffy and Hall 2008; Hall et al. 2009; Wolinska et al. 2011). Whereas the young-of-the-year cohorts of planktivorous fish are the main cause of *Daphnia* mortality during late spring and early summer (e.g., Vijverberg and Richter 1982). This could promote the evolution of inducible life-history traits in this system. For *Daphnia* living with multiple stressors such as predation and parasitism it pays off to invest resources into the most dominant threat at a given time. Thus, when fish predators are the most important threat it pays off to invest in inducible morphological and life-history defenses. However, when parasites increase in prevalence and become the dominant threat, the best strategy may be to shut off the inducible defenses and devote more energy and resources into somatic maintenance and the immune system.

The mechanisms through which inducible defenses increase *Daphnia*'s susceptibility to parasites are unknown. One potential explanation would be that the changes in prey behavior, as induced by predators, alter the contact rate with parasites. For example, damselfly larvae reduce grooming when fish are present, as fish cue on larval movement. Consequently, larvae become heavily infected by parasitic mites, which are normally avoided by active grooming (Baker and Smith 1997). Another case is the freshwater snail, which retreats into the safety of its shell when attacked by predators. To do this, however, the snail must expel its blood. As blood contains many components of the immune system, this type of predator avoidance reduces the snails' ability to defend against potential parasites (Rigby and Jokela 2000). Furthermore, tadpoles that are exposed to fish predators are more susceptible to trematode infection than unexposed tadpoles. This is caused by a reduction in the tadpoles' activity in the presence of predators, which consequently increases their proximity to infective cercaria (Thiemann and Wassersug 2000). Finally, a well-documented *Daphnia* behavioral defense against fish predation is to reside in deeper and darker portions of the water column during the day (De Meester et al. 1995). However, this behavior increases the risk of infection from sediment that contains parasite spores (known as the "deep trouble hypothesis," Decaestecker et al. 2002). In our experiment, however, the potential behavioral differences between induced and noninduced individuals were excluded by using small (5 mL to 30 mL) vessels. Thus, it seems not to be the case that predator-defended *Daphnia* suffered higher vulnerability to parasites, because of their increased encounter rates with parasite spores.

Another cause for the increased infection of predator-defended morphs could be due to an impaired immune system. For example, beetles exposed to predators were more susceptible to subsequent parasite infection, consistent with the observation in the laboratory that predator exposure weakens the beetles'

immune response (Ramirez and Snyder 2009). The authors argued that the weakened immune system was likely a result of energetic costs associated with inducible defenses and/or the abandonment of optimal feeding sites. In general, a variety of environmental stressors, for example food depletion or predator exposure, are known to suppress immune function in different animal systems (e.g., Raberg et al. 1998; Rigby and Jokela 2000; Lee et al. 2006; Zysling and Demas 2007). Thus, investment into defenses against life-threatening predators may reduce the effectiveness of the prey's immune system, and consequently result in an enhanced vulnerability to infection. An impaired immune system is not an unlikely explanation of our results, especially given that recent work on *D. magna* found that immune function, indirectly measured as phenoloxidase activity, was lower when *Daphnia* were exposed to fish kairomones (Pauwels et al. 2010). However, the potential trade-offs within the *Daphnia* host regarding resource allocation between inducible defenses and immune function requires further investigation.

Our study calls to attention that the costs of inducible defenses may have been explored in too simplified context. The here observed costs to predator-defended *Daphnia* are substantial as the fitness reduction via parasitism is high; in the first clutch infected *Daphnia* produce half the number of offspring as healthy animals and infected *Daphnia* stop reproducing after their second or third clutch (Ebert et al. 2000; Lohr et al. 2010). Thus, as we found the defended morphs to be more susceptible to parasitism, this relatively large cost of parasitism may produce a strong selection pressure to forgo the formation of defenses when predators are absent. The detection of such environmentally relevant costs increases our understanding of the adaptive value and evolution of phenotypic plasticity.

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GENERAL DISCUSSION

6.1. Discussion

By combining field sampling, molecular techniques and laboratory experiments, this PhD project aimed to obtain a better understanding of population structure and host-parasite interactions in the *D. longispina* hybrid complex.

6.1.1. High resolution genotyping in *D. longispina* hybrid complex

Before any conclusions can be drawn on the population structure in the *D. longispina* hybrid complex, accurate methods for classification of individuals need to be applied. First, in Chapter 2, in order to corroborate the taxonomic assignment of individuals from field samples, I used the information from reference clones genotyped by allozymes, mtDNA and microsatellite markers. I detected a nearly perfect correspondence in assignments between my specific set of microsatellite loci and other genetic markers, which is consistent with recent findings for *Daphnia* individuals from other geographical regions based on a different selection of microsatellites (Thielsch et al., 2009; Dlouhá et al., 2010). Furthermore, field collected *Daphnia* were consistently assigned to major taxonomic groups by three independent statistical approaches (factorial correspondence analysis and two Bayesian methods). Therefore, in the present PhD project, I could use this set of high resolution

microsatellite markers (15 loci in Chapter 2, 10 loci in Chapters 3 and 4, and 15 loci in Chapter 5).

In Chapter 2, I reported the year-to-year changes in the taxon composition of *D. longispina* assemblages. Most strikingly, in one of three studied lakes (i.e. Feldmochinger See), the F1 hybrids were completely replaced by parental *D. galeata* in the following year. Previous studies have shown the fluctuations in the relative taxon frequencies in this species complex (e.g. Spaak and Hoekstra, 1997; Keller and Spaak, 2004); however, such dramatic changes from complete dominance by F1 hybrids to a complete dominance of *D. galeata* as observed in my study seem to be quite rare. While cyclical changes in taxon frequency can be easily explained by repeatable, seasonal fluctuations in environmental conditions, such as temperature (e.g. Benndorf et al., 2001) and predation pressure (e.g. Spaak *et al.*, 2000), a complete replacement must have other reasons. In contrast, I observed that the taxonomic composition of Feldmochinger See changed dramatically across samples taken at the same time point in subsequent years (in spring), just after populations hatched from diapausing eggs. Such a rapid replacement (i.e. hybrids replaced by one parental taxon) not correlated with seasonal changes has not been reported in the *D. longispina* hybrid complex before. The possible reason could be a pure taxon effect on the survival of overwintering asexual clones. Asexual clones of parental and hybrid taxa could respond differently to winter conditions (Rellstab and Spaak, 2009). An alternative explanation for this replacement could be the size of the dormant egg bank. It has been shown that the egg bank of parental species is much larger than that of hybrid taxa (Keller and Spaak, 2004; Keller et al., 2007). Because the population with the larger egg bank is expected to start with more hatchlings at the beginning of the new growing season, it could easily out-compete the hybrids, which have lower contribution of hatchlings from the sediment (Keller et al., 2007). In addition, the selection

pressure between years might have changed significantly. For example, in two recent studies (Wolinska et al., 2006; Brede et al., 2009), the observed changes in *D. longispina* taxon composition were related to long-term selective directional rather than seasonal change in environmental conditions, i.e. increasing lake trophy (Brede et al., 2009) and changes in parasite selection pressure (Wolinska et al., 2006). However, the changes in taxon composition were relatively slow (lasting a few to dozen years), compared to the rapid change as observed in Chapter 2. Further studies are needed to explore the reasons for this rapid taxon replacement.

In Chapter 2, I found that F1 hybrids have lower genotypic diversity than parental populations, as expected because of a predominantly clonal reproduction in hybrid populations (Keller et al., 2007). However, genotypic diversity was compared between the taxa originating from eight different water bodies, so it could not be excluded that the observed patterns were partially caused by habitat differences. Therefore, in Chapter 3, I compared the genotypic diversity between co-occurring taxa within a single water body. My data indicated that genotypic diversity in F1 hybrids was much lower than in parental species, strongly supporting the existence of reproductive incompatibilities between the parental genomes of hybridizing species in the *D. longispina* complex.

By applying a fine-scale sampling design, Chapter 3 highlighted the significant changes in *Daphnia* taxon and clonal composition both on temporal and spatial scale, in the *Daphnia* communities inhabiting two water bodies. Surprisingly, my data showed spatial genetic differentiation on such a small scale, like within a single water body. Moreover, I observed fast replacement of dominant clones in a very short time period (within one or two *Daphnia* generation times). The observed pattern can be explained by the impact of environmental

heterogeneity in space and time: previous experimental studies on the *D. longispina* complex have shown that relative taxon and clonal fitness varies with food quality (e.g. Von Elert, 2004; Seidendorf et al., 2007), temperature (e.g. Wolf and Weider, 1991) and predation pressure (e.g. Spaak et al., 2000; Spaak and Boersma, 2006). Although I detected significant spatial genetic differentiation, certain clones were present in substantial frequencies at sampling stations separated by several kilometres. Obviously, such successful genotypes can reach high densities and occupy vast areas within the water body despite the variation in selection pressure across sampling sites. Most likely, these common clones could overwinter in the water body, which allows them to compete with other genotypes for extended periods of time.

6.1.2. Parasite-driven selection

One possible factor driving the changes in *Daphnia* taxon and clonal composition could be a selection pressure as induced by parasites. As showed in Chapter 3, the clonal diversity of *D. galeata* increased with time, since a few dominant clones were replaced by a higher number of less common clones. Maybe, these dominant clones could have been infected, as the parasite prevalence in these *Daphnia* host populations was very high (Chapter 4). Therefore, a loss in selective advantage for the dominant clones may have been due to parasite selection acting in a negative frequency-dependent manner (see Jokela et al., 2009). In Chapter 4, I detected significant differences in clonal composition between random and *Caullerya*-infected parts of the host population. Similar differences were also detected in *Daphnia* populations infected with another parasite, *Metschnikowia*, which was earlier thought to be a generalist (e.g. Stirnadel and Ebert, 1997). This suggests that parasite-driven selection may operate in natural *Daphnia* populations (e.g. Little and Ebert, 1999; Duncan and Little, 2007; Wolinska and Spaak, 2009), as parasites influence the clonal structure of host population.

6.1.3. Cost of inducible defences

When we consider the changes of taxon and clonal composition in natural *D. longispina* hybrids complex; parasites are not the only selection pressure, predators have been also shown to be an important driving factor (e.g. Spaak and Hoekstra, 1997; Wolinska et al., 2007b). Importantly, both pressures (i.e. predators and parasites) frequently co-occur in *Daphnia* population of European lakes. Therefore, it may be very common that the defended *Daphnia* (i.e. after being exposed to chemical cues released by predator) have to encounter their parasites. In Chapter 5, I simulated this situation and exposed the defended *Daphnia* to the spores of yeast parasite, *Metschnikowia*. The proportion of successful infections and the numbers of parasite spores were higher for the defended than undefended morphs. Strikingly, the pattern was consistent across five tested clones (two *D. galeata* and three *D. galeata* × *D. longispina* hybrid clones), suggesting this could be a general pattern in a *Daphnia* system. The high virulence of parasites can largely reduce the fitness of *Daphnia* host population. For example, *Metschnikowia*-infected *Daphnia* only produce half the number of offspring as healthy ones in the first clutch and infected *Daphnia* stop reproducing after their second and third clutch (e.g. Ebert et al., 2000; Lohr et al., 2010a). Therefore, higher susceptibility to parasitism of defended morphs would be the substantial cost of inducible defences against predator.

In conclusion, I investigated population structure of natural *D. longispina* hybrid complex (Chapters 2-4) under three different selection pressures (hybridization, parthenogentic reproduction and parasites). Moreover, a previously unknown and environmentally relevant cost to *Daphnia* inducible defences, parasitism, was demonstrated in Chapter 5.

6.2. Future Directions

6.2.1. Hybridization in novel habitats

It has been shown that parental and hybrid taxa from the *D. longispina* complex co-occur in many European lakes (e.g. Keller et al., 2008), and hybrids are sometimes the most abundant taxon (e.g. Chapter_2, Wolinska et al., 2006; Keller et al., 2007). The dominance of hybrids could be explained by their successful clonal reproduction, which then overcomes problems related with potential sexual incompatibilitieas (e.g. Lynch and Gabriel, 1990). This was confirmed in Chapters 2 and 3, where I observed the lower genotypic diversity in hybrids than in parental species, as expected from the high rate of asexual reproduction. However, most of previous studies were carried out in the large natural lakes (or human-made reservoirs), where hybrid clones can be propagated for a long time through asexual reproduction, without a necessity of going through sexual recombination in winter (e.g. Keller et al., 2007). To explore potential differences in clonal survival, I investigated in Chapter 2 several small and shallow quarry lakes, where *Daphnia* hybrid communities are forced to go through sexual reproduction at the end of each growing season. In contrast to rather stable taxa composition in large-lake dataset, year-to-year changes in the taxon composition of *D. longispina* assemblages were detected in two out of three artificial lakes; in Feldmochinger See, for example, the F1 hybrids which dominated the community in 2008 were completely replaced by parental *D. galeata* in 2009. Moreover, *D. galeata* was then again replaced by F1 hybrids in 2010 (Yin et al., unpublished data). The twofold complete taxon replacement suggests an advantage of hybrids under certain conditions and that some *D. longispina* communities inhabiting artificial lakes need to be re-established every year in spring.

Further studies to investigate how hybrid-specific traits could facilitate colonization of novel habitats (in a sense of spring re-establishment) are needed. Moreover, it remains to be tested,

if population re-establishment happens through migration or from the sexual egg bank (i.e. from the lake sediment). We have established ideal field sites (i.e. small and shallow quarry lakes in and around Munich) that offer the opportunity to study *Daphnia* hybrid communities that are forced to go through sexual reproduction at the end of each growing season. We have sampled 15 such habitats and detected *D. longispina* communities in 10 of them (Yin et al., unpublished data collected in 2008). We have collected spring samples (when a pelagic population of *Daphnia* gets re-established) from four succeeding years (2008-2011). These samples are currently being genotyped and will be later tested for new hybridization events and the establishment of hybrids by migrants, respectively. Tracing the hybrid clonal lineages, both across space (i.e. different habitats) and time (i.e. different years), will contribute to answering the question of hybrid success in novel habitats (e.g. Lewontin and Birch, 1966; Arnold and Martin, 2010).

6.2.2. Clonal diversity and environments

In cyclical parthenogenes, the asexual life cycle can last for a long time. During this parthenogenetic phase, clonal selection can erode the initial genetic diversity, a process known as “clonal erosion” (De Meester et al., 2006; Vanoverbeke and De Meester, 2010). For example, a decrease in clonal diversity has been detected in *D. magna* and *D. pulex* populations inhabiting temporary ponds, during the course of a single growing season (Ruvinsky et al., 1986; Carvalho and Crisp, 1987) and similar patterns were reported for rotifers (e.g. Ortells et al., 2006) and aphids (Sunnucks et al., 1997). In Chapter 3, I found clonal diversity remaining roughly constant in one water body and even increasing in another water body in succeeding samples. Surprisingly, the number of rare clones increased with time suggesting that “clonal erosion” did not operate at the end of the growing season.

Unfortunately, our samples were only collected from the middle (summer) towards the end of a growing season (autumn), rather than from the beginning of growing season (spring) precluding a solid evidence for an increase in diversity. Clearly, one should track changes in *Daphnia* population structure from the very beginning (spring) till the end of the growing season (end of autumn) in future studies. Moreover, one should stick to applying a sufficiently high number of microsatellite makers to be able to trace true clonal lineages. Therefore, we have now been collecting monthly samples from ten small and shallow quarry lakes in and around Munich (April - October 2011). The samples will be genotyped (at 15 microsatellite loci) in the near future to create a data set allowing for testing the "clonal erosion" hypothesis.

A potential explanation for the increasing clonal diversity over time, as observed in Chapter 3, would be that some common clones may invest into sexual rather than parthenogenetic reproduction at the end of season. However, this was not the case in our data set; as the proportion of ephippial females and males was very low (Chapter 3). An alternative explanation could be that the most common clones would be tracked by parasites, resulting then in their drop in frequencies. Finally, the presence of rare clones at the end of growing season might be explained by their selective advantage. These rare clones may not have been favoured during stratified summer conditions because of relatively lower fitness, while they may be better adapted to low food and low temperature (i.e. conditions specific for the end of a growing season). For example, a recent study in the same water reservoir detected the presence of a genetically differentiated hypolimnetic population of *Daphnia* (Seda et al., 2007a) with life-history traits different from their epilimnetic counterparts (Machacek and Seda, 2008). Thus, clones originating from the deep layers, where the water is colder and less nutrient-rich, may have an advantage at the end of the growing season. Also here future experiments are required to test if some specific environmental conditions, such as food

availability, could potentially favour previously rare clones. This would help in better understanding of the clonal dynamics in different habitats.

6.2.3. Molecular mechanisms for costs of inducible defences

The mechanisms why predator-induced *Daphnia* have increased susceptibility to parasites, as shown in Chapter 5, are still unknown. One potential explanation could be that the induced *Daphnia* change their behaviour (e.g. De Meester et al., 1995) and thus have a greater chance to become exposed to parasites. The "deep trouble" hypothesis predicts that *Daphnia* suffer less from visually hunting fish by residing in deeper and darker portions of water column. At the same time, however, this behaviour increases the risk of parasitic infections when the *Daphnia* are exposed to pond sediments containing parasite spores (Decaestecker et al., 2002). One could argue that, in my experiment, the parasite spores could possible settle down to the bottom of jars. Thus, the induced *Daphnia* could have a greater contact rate with parasites if they stay in the bottom of the water vessels in consequence of their typical behavioural response to fish cues. In our study, however, behavioural changes and higher vulnerability to infection in the induced daphnids cannot be explained by a higher encounter rate with parasite spores. Firstly, both the induced and non-induced individuals were maintained in small vessels (5-30 ml media), without a possibility to perform vertical migrations and, secondly, the water in the jars was steered daily. Moreover, in another *Daphnia* system, induction of defences against another predators (larvae of the invertebrate phantom midge, *Chaoborus*), also increased the prevalence of infection and number of spores upon death (Duffy et al., 2011). The authors explained this phenomenon by the fact that the *Chaoborus*-induced *Daphnia* grow larger (as previously shown e.g. Stibor and Lüning, 1994). Then, growing larger enhances *Daphnia* susceptibility to parasites because larger hosts contact more infectious spores while feeding (Hall et al., 2007). According to this prediction, the fish

kairomones should decrease *Daphnia* susceptibility to parasites, as fish kairomones induce *Daphnia* to reproduce earlier, at smaller size (e.g. Stibor and Lüning, 1994; Rinke et al., 2008). Since my results (Chapter 5) showed the opposite pattern, I can reject this explanation. Therefore, it seems that the observed increased infection of predator-induced morphs in my study could be due to their impaired immune system. Suppressed immune functions resulting from multiple stressors (like food depletion or predator exposure) have been already shown in different animal systems (e.g. Raberg et al., 1998; Rigby and Jokela, 2000; Lee et al., 2006) and could potientially explain also my findings.

The recent release of the *Daphnia pulex* genome (Colbourne et al., 2011) creates the opportunity to precisely identify candidate genes and reveal the molecular signalling pathways of *Daphnia* responses to either stressor: predation and parasitism. Many candidate genes, such as cyclophilin and Hox3, have been shown to be significantly up-regulated by predator cues (e.g. Schwarzenberger et al., 2009; Miyakawa et al., 2010; Spanier et al., 2010). At the same time, some of the classical housekeeping genes, such as alpha tubulin and glyceraldehyde-3-phosophate dehydrogenase, were significantly down-regulated (e.g. Spanier et al., 2010). In addition, juvenile hormone pathways and insulin signalling pathways were proven to be up-regulated during the activation of inducible defences (Miyakawa et al., 2010). Especially, it has been shown that hormonal pathways are involved in physiological regulation following morphogenesis in many insect species (e.g. Nijhout, 1999; Truman et al., 2006). By comparison of *D. pulex* gene sequences with homologues genes from other arthropods, some genes (such as genes of peptidoglycan recognition proteins and gram-negative binding proteins) as well as a TOLL pathway (the important pathway in the innate immune system for both invertebrates and vertebrates), were identified to be related with the *Daphnia* immune system (McTaggart et al., 2009). However, in a recent experimental study

on *D. magna*, some candidate genes from this list (McTaggart et al., 2009) showed nosignificant over-expression level in response to parasites (Decaestecker et al., 2011). Another study on *D. magna* described that the immune function (measured by phenoloxidase activity) was lower when daphnids were exposed to fish kairomones. However, the only focus of this study was looking at changes in phenoloxidase activity while the molecular mechanisms remained unexplored (Pauwels et al., 2010). So far, studies are missing focussing on relationships between induction (i.e. inducible defenses) and the immune system pathways. If their molecular pathways overlap, responses to induction could trigger changes in immune pathways. Further investigations of the molecular pathways of both are required, to enhance our knowledge about how epidemiology can be integrated into the concept of phenotypic plasticity.

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APPENDIX A

Table 2.S1. List of 49 reference genotypes, including their sampling location and genetic identification by different marker systems.

ID	Allozyme-type	Morph	Microsatellites	Mt-DNA (12S and/or Cytb)	Lake	Country
Ba16	Cuc	Cuc	Cuc	Cuc	Bansee	Germany
Cuc33	Cuc	Cuc	Cuc	Cuc	Tjeukemer	Netherlands
CucTS_S8	Cuc	Cuc	Cuc	NA	Thaler See	Germany
CV50	Cuc	Cuc	Cuc	Cuc	Tjeukemer	Netherlands
SpecSchw	NA	Cuc	Cuc	Cuc	unknown	Germany
Tc3	Cuc	Cuc	Cuc	Cuc	Thaler See	Sweden
TS64	Cuc	Cuc	Cuc	Cuc	Thaler See	Germany
TSBr	Cuc	Cuc	Cuc	Cuc	Thaler See	Germany
Car16	NA	Gal	Gal	Gal	Lough Caragh	Irland
DgCZ11	Gal	Gal	Gal	Gal	unknown	Tschechien
G100	Gal	Gal	Gal	NA	Tjeukemer	Netherlands
G44	Gal	Gal	Gal	Gal	Grote brekken	Netherlands
Gal1	Gal	Gal	Gal	Gal	Ismaninger Teich	Germany
GalB2	NA	Gal	Gal	Gal	unknown	North America
O2	Gal	Gal	Gal × Lon	Gal	Obinger See	Germany
O31	Gal	Gal	Gal	Gal	Saidenbachtalsperre	Germany
T1-19	Gal	Gal	Gal	NA	Obinger See	Germany
AH25	Lon	Hyl	Lon	Lon	Klostersee	Germany
Am1	Lon	Ros	Lon	Lon	pond near Schondorf	Germany
Am2	Lon	Hyl× Ros	Lon	Lon	pond near Schondorf	Germany
ANK2	Lon	Hyl	Lon	Lon	Klostersee	Germany
AP14	Lon	Hyl	Lon	Lon	Pelhamer See	Germany
AS4	Lon	Hyl× Ros	Lon	Lon	Seeleitensee	Germany
B016	Lon	Hyl× Ros	Lon	Lon	Seeleitensee	Germany
D1	Lon	Ros	Lon	Lon	Ismaninger Teich	Germany
D9	Lon	Ros	Lon	Lon	Ismaninger Teich	Germany
Fehya	Lon	Hyl	Lon	Lon	Ferchensee	Germany
Fr8	Lon	Hyl	Lon	Lon	Frauensee	Austria
Fr11	Lon	Hyl	Lon	Lon	Frauensee	Austria
Frauhya	Lon	Hyl	Lon	NA	Frauensee	Austria
H7	Lon	Hyl	Lon	NA	Bodensee	Germany
H106	Lon	Hyl	Lon	Lon	Hartsee	Germany
H114	Lon	Hyl	Lon	Lon	Hartsee	Germany
H130	Lon	Hyl	Lon	Lon	Hartsee	Germany
H147	Lon	Hyl	Lon	Lon	Hartsee	Germany
HyaStich	Lon	Hyl	Lon	Lon	Bodensee	Germany
JBR84	Lon	Hyl	Lon	Lon	Brunnsee	Germany
Ka12	Lon	Hyl × Ros	Lon	Lon	Kalbelesee	Austria
L6	Lon	Hyl	Lon	Lon	Lautersee	Germany
O1	Lon	Hyl	Lon	Lon	Obinger See	Germany
O84B23	Lon	Hyl	Lon	Lon	Brunnsee	Germany

RoseaR9	Lon	Ros	Lon	Lon	pond near Plön	Germany
Su1	Lon	Ros	Lon	Lon	Suttensee	Germany
Su15	Lon	Ros	Lon	Lon	Suttensee	Germany
T6-20	Lon	Hyl	Lon	NA	Saidenbachtalsperre	Germany
W18	Lon	Hyl	Lon	NA	Walchensee	Germany
T40-16	Gal × Lon	Gal × Hyl	Gal × Lon	NA	Saidenbachtalsperre	Germany
Labgxh	Gal × Lon	Gal × Hyl	Gal × Lon	Gal	Klostersee	Germany
Dgc1	Cuc × Gal	Cuc × Gal	Cuc × Gal	Cuc	unknown	Netherlands

Cuc, Gal, Lon, Hyl and Ros are the abbreviations for *D. cucullata*, *D. galeata*, *D. longispina* with *hyalina* and *rosea* morphs, respectively; NA: data not available.

Table 2.S2. Results of the computation of the most likely number of clusters (*K*) in STRUCTURE analysis. L(K) and ΔK are likelihoods from 15 simulations. The most likely number of clusters (*K* = 5) was chosen by highest ΔK.

K	L(K)	ΔK
2	-16476.2	
3	-15156.7	2.31
4	-13913.3	2.08
5	-12891.3	9.10
6	-12433.2	0.98
7	-11950.0	1.00
8	-11566.4	4.60

Table 2.S3. Private alleles at 15 microsatellite loci in *D. galeata* and *D. longispina* as defined by CONVERT. Calculations were based on the selection of MLGs from 2008 field samples and reference genotypes. A: number of alleles per locus, A_T: number of alleles per locus and taxon, A_S: number of private alleles per locus, A_r: allelic richness per locus. A_P: the abundance of private alleles per locus. The potentially species-specific alleles (i.e. $A_P > 5\%$ for both taxa) are indicated in bold.

Locus ID	A	Taxon [a]	A_T	A_S	A_r	List of private alleles - fragment size (bp)	A_P (%)
Dgm105	16	Lon	15	11	11.0	174 180 181 185 186 188 189 191 192 197 199	23.8
		Gal	5	1	5.0	190	1.6
Dgm109	23	Lon	21	14	17.1	234 235 245 246 247 249 250 252 254 255 257 258 259 265	58.3
		Gal	9	2	10.0	266 268	1.6
Dgm112	11	Lon	9	4	7.7	107 109 114 121	95.6
		Gal	7	2	7.0	119 132	1.0
Dp196	4	Lon	3	1	3.2	116	2.5
		Gal	3	1	4.0	114	21.7
Dp281	6	Lon	5	3	4.6	73 74 76	24.2
		Gal	3	1	4.0	77	1.0
Dp512	10	Lon	10	7	7.2	130 131 134 136 137 138 140	73.4
		Gal	3	0	3.0	none	0
SwiD1	27	Lon	24	19	17.9	113 115 117 118 119 121 125 126 128 136 137 141 143 146 148 149 150 151 153	80.4
		Gal	8	3	7.0	122 124 127	4.1
SwiD2	17	Lon	12	3	8.4	162 164 170	1.9
		Gal	14	5	11	172 174 180 186 194	18.1
SwiD5	17	Lon	10	7	7.1	129 130 135 136 138 140 155	**56.1**
		Gal	10	7	10	150 152 154 156 162 164 166	**52.7**
SwiD7	9	Lon	8	5	5.4	153 157 163 168 171	**23.7**
		Gal	4	1	4.0	159	**43.8**
SwiD8	7	Lon	3	1	9.4	119	0.6
		Gal	6	4	8.0	140 149 150 154	95.9
SwiD10	19	Lon	12	8	9.5	180 181 183 185 188 191 192 206	**8.5**
		Gal	11	7	14.0	182 194 200 201 202 216 225	**73.2**
SwiD12	13	Lon	9	3	8.2	114 118 130	**42.5**
		Gal	10	4	8	110 117 119 121	**63.4**
SwiD14	7	Lon	7	3	6.9	182 187 189	7.9
		Gal	4	0	4.0	none	0

SwiD15	15	Lon	10	8	2.5	70 76 77 79 81 83 85 86	**98.1**
		Gal	7	5	6.0	88 90 92 96 98	**43.3**
Total	201	Lon	158	97			
		Gal	104	43			

[a] the taxon membership was defined by the NewHybrids software based on the allelic variation at 15 microsatellite loci, Gal and Lon are the abbreviations for *D. galeata* and *D. longispina*, respectively.

APPENDIX B

Table 3.S1. Sampling date information.

	Římov			Vír		
Sample	Sampling date	Interval (days)	Temperature[a] (°C)	Sampling date	Interval (days)	Temperature[a] (°C)
t	15 Sep 2009		16.6	14 Sep 2009		17.8
		15			14	
t+1	30 Sep 2009		16.4	28 Sep 2009		16.9
		16			17	
t+2	16 Oct 2009		12.6	15 Oct 2009		14.1
		26			28	
t+3	11 Nov 2009		8.4	12 Nov 2009		10.3
		30			27	
t+4	11 Dec 2009		6.2	09 Dec 2009		8.3

[a] the temperature was measured at the depth of 2 meters at *dam*.

Table 3.S2. Clonal diversity of *Daphnia* populations, as calculated from 10 microsatellite loci.

Reservoir	Station	Sampling date	Taxon [a]	N [b]	N [c]	MLG	MLG/N [c]
Římov	*dam*	*t*	*D. galeata*	93	91	48	0.53
		t+1		91	91	31	0.34
		t+2		88	87	58	0.67
		t+3		88	87	65	0.75
		t+4		78	60	46	0.77
	middle	*t*		94	94	42	0.45
		t+1		85	82	32	0.39
		t+2	*D. galeata*	94	94	50	0.53
		t+3		85	82	52	0.63
		t+4		94	93	50	0.54
	upper	*t*		94	93	29	0.31
		t+1	*D. galeata*	89	87	53	0.61
		t+2		88	87	46	0.53
		t+3		93	92	50	0.54
Vír	*dam*	*t*	*D. galeata*	46	46	31	0.67
			F1 hybrids	19	19	12	0.63
			D. longispina	13	13	10	0.77
			unidentified	7	6	4	nc
		t+1	*D. galeata*	56	56	40	0.71
			F1 hybrids	22	22	12	0.55
			D. longispina	7	7	6	nc
			unidentified	7	5	3	nc
		t+2	*D. galeata*	15	15	10	0.67
			F1 hybrids	19	19	10	0.53
			D. longispina	35	35	27	0.77
			unidentified	13	13	7	0.54
		t+3	*D. galeata*	24	23	21	0.91
			F1 hybrids	17	17	12	0.71
			D. longispina	42	41	40	0.98
			unidentified	5	5	5	nc
		t+4	*D. galeata*	29	28	22	0.79
			F1 hybrids	27	27	10	0.37
			D. longispina	27	27	27	1.00
			unidentified	7	7	3	nc
	middle	*t*	*D. galeata*	36	36	30	0.83
			F1 hybrids	22	22	8	0.36
			D. longispina	26	26	20	0.77
			unidentified	5	5	5	nc
		t+1	*D. galeata*	23	23	17	0.74
			F1 hybrids	37	37	16	0.43
			D. longispina	15	15	7	0.47
			unidentified	12	12	7	0.58
		t+2	*D. galeata*	61	61	42	0.69
			F1 hybrids	21	21	10	0.47
			D. longispina	4	4	4	nc
			unidentified	6	5	5	nc
		t+3	*D. galeata*	55	51	26	0.51

		F1 hybrids	8	8	7	nc
		D. longispina	13	12	10	0.83
		unidentified	10	6	5	nc
	t+4	*D. galeata*	40	38	33	0.89
		F1 hybrids	31	31	16	0.52
		D. longispina	11	11	5	0.45
		unidentified	7	6	5	nc
upper	*t*	*D. galeata*	70	67	48	0.72
		F1 hybrids	13	13	3	0.23
		D. longispina	3	3	2	nc
		unidentified	5	1	1	nc
	t+1	*D. galeata*	66	65	44	0.68
		F1 hybrids	19	19	4	0.21
		D. longispina	2	2	1	nc
		unidentified	4	3	3	nc
	t+2	*D. galeata*	79	78	46	0.59
		F1 hybrids	13	13	7	0.54
		D. longispina	1	1	1	nc
		unidentified	0			
	t+3	*D. galeata*	73	68	36	0.53
		F1 hybrids	8	8	8	nc
		D. longispina	7	7	7	nc
		unidentified	3	2	2	nc
	t+4	*D. galeata*	47	47	31	0.66
		F1 hybrids	8	8	6	nc
		D. longispina	20	20	14	0.70
		unidentified	4	4	4	nc

[a] the taxon membership was defined by the NewHybrid software based on the allelic variation at 10 microsatellite loci (only a few individuals were classified to the backcross taxa, and those are not included here); N [b], total number of individuals; N [c], number of individuals excluding missing data (in case of *D. longispina* individuals from the Vír reservoir, individuals with the missing data at the locus SwiD2 are included); MLG, number of unique multi-locus genotypes; nc, not calculated.

Table 3.S3. Factors influencing the taxon composition in Vír.

Model	Residual DF	Residual Deviance	DF	LR statistic	*P*
time and space	24	2232.2			
time, space and their interaction	22	2226.5	2	5.73	0.06
space	26	2246.3			
time and space	24	2232.2	2	14.09	**< 0.001**
time	26	2354.9			
time and space	24	2232.2	2	122.71	**< 0.001**

Table 3.S4. Factors influencing the clonal composition within taxa for each reservoir.

Taxon (reservoir)	Model	Residual DF	Residual Deviance	DF	LR statistic	*P*
D.galeata (Římov)	time and space	55	801.1			
	time, space and their interaction	50	787.2	5	13.94	**0.016**
	space	60	922.7			
	time and space	55	801.1	5	121.64	**< 0.001**
	time	60	980.9			
	time and space	55	801.1	5	179.78	**< 0.001**
D.galeata (Vír)	time and space	48	1110.9			
	time, space and their interaction	44	1089.8	4	21.02	**< 0.001**
	space	52	1128.7			
	time and space	48	1110.9	4	17.86	**0.001**
	time	52	1128.2			
	time and space	48	1110.9	4	17.34	**0.002**
F1 hybrids (Vír)	time and space	12	232.8			
	time, space and their interaction	10	221.9	2	10.89	**0.004**
	space	14	247.2			
	time and space	12	232.8	2	14.46	**< 0.001**
	time	14	242.4			
	time and space	12	232.8	2	9.69	**0.008**
D. longispina (Vír)	time and space	60	527.4			
	time, space and their interaction	55	512.5	5	14.92	**0.011**
	space	65	612.9			
	time and space	60	527.4	5	85.53	**< 0.001**
	time	65	599.0			
	time and space	60	527.4	5	71.65	**< 0.001**

APPENDIX C

Table 4.S1. Sampling date information.

	Římov		Vír	
Sample	Sampling date	Temperature [a] (°C)	Sampling date	Temperature [a] (°C)
t	15 Sep 2009	16.6	14 Sep 2009	17.8
t+1	30 Sep 2009	16.4	28 Sep 2009	16.9
t+2	16 Oct 2009	12.6	15 Oct 2009	14.1
t+3	11 Nov 2009	8.4	12 Nov 2009	10.3
t+4	11 Dec 2009	6.2	09 Dec 2009	8.3

[a] the temperature was measured at depth of 2 meter at *dam*.

Table 4.S2. Comparison of genetic diversity between random and infected samples (*Caullerya*-infected in Římov and *Metschnikowia*-infected in Vír), at three different stations (*dam*, *middle* and *upper*) and two or three sampling dates (*t+1*, *t+2* and *t+3*), calculated based on 10 microsatellite loci.

			Parasite prevalence (%)			Random		Infected	
Reservior	Station	Sampling date	*Caullerya*	*Metschnikowia*	Host taxon[a]	N[b]	MLG/N[b]	N[b]	MLG/N[b]
Římov	*dam*	*t*	1.2	not detected	NA				
		t+1	8.4	not detected	*D. galeata*	91	0.34	43	0.37
		t+2	4.4	not detected	*D. galeata*	87	0.67	42	0.64
		t+3	2.0	not detected	*D. galeata*	87	0.75	36	0.81
		t+4	not detected	not detected	NA				
	middle	*t*	2.6	not detected	NA				
		t+1	11.7	not detected	*D. galeata*	82	0.39	45	0.33
		t+2	10.0	not detected	*D. galeata*	94	0.54	45	0.67
		t+3	1.8	not detected	*D. galeata*	82	0.63	29	0.69
		t+4	0.6	not detected	NA				
	upper	*t*	2.2	not detected	NA				
		t+1	7.4	not detected	*D. galeata*	87	0.61	42	0.69
		t+2	17.8	not detected	*D. galeata*	87	0.53	45	0.51
		t+3	7.4	not detected	*D. galeata*	92	0.54	41	0.66
Vír	*dam*	*t*	not detected	3.8	NA				
		t+1	0.7	not detected	NA				
		t+2	not detected	9.1	*D. galeata*	15	nc	4	nc
					hybrids	22	0.59	18	nc
					D. longispina	52	0.65	24	0.29
		t+3	not detected	7.6	*D. galeata*	23	0.91	6	nc
					hybrids	21	0.76	13	nc
					D. longispina	44	0.98	28	0.68

	t+4	not detected	1.3	NA				
middle	*t*	1.5	not detected	NA				
	t+1	not detected	1.3	NA				
	t+2	not detected	15.6	*D. galeata*	61	0.69	20	0.75
				hybrids	21	0.48	18	nc
				D. longispina	10	nc	9	nc
	t+3	not detected	15.2	*D. galeata*	53	0.51	20	0.9
				hybrids	11	nc	11	nc
				D. longispina	21	0.76	14	nc
	t+4	not detected	4.5	NA				
upper	*t*	2.8	3.2	NA				
	t+1	0.7	2.0	NA				
	t+2	not detected	3.3	*D. galeata*	79	0.59	30	0.67
				hybrids	13	nc	13	nc
				D. longispina	1	nc	4	nc
	t+3	not detected	7.9	*D. galeata*	69	0.54	29	0.52
				hybrids	10	nc	5	nc
				D. longispina	8	nc	10	nc
	t+4	not detected	4.6	NA				

[a] the taxon membership was defined by the NewHybrid software based on the allelic variation at 10 microsatellite loci; [b] number of genotyped individuals in random samples excluding missing data; MLG, unique multi-locus genotypes; NA, not analysed (due to low parasite prevalence); nc, not calculated (due to low sample size).

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